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O impacto do biofilme no perfil de susceptibilidade aos antibióticos e a caracterização dos principais determinantes de sua resistência em *Staphylococcus* spp.

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Orientador: Dr. Pedro Alves d'Azevedo

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RESUMO

Staphylococcus são microrganismos comensais e habitantes da pele humana que emergiram de maneira oportunista, principalmente em infecções associadas à formação de biofilme. O biofilme é uma comunidade de bactérias vivendo em organizadas estruturas e sua presença em infecções crônicas está associada a sua resistência contra antibióticos utilizados para tratamento destas infecções. Essa resistência pode ocorrer principalmente devido à fisiologia e estrutura do próprio biofilme. Este estudo foi desenvolvido com métodos fenotípicos para avaliar a formação de biofilme em *Staphylococcus* spp, e com a utilização de ferramentas moleculares para avaliar a expressão de genes associados à formação e regulação destes biofilmes. Em ambos os casos, as avaliações se basearam em biofilmes expostos ou não a antibióticos. Foram selecionados isolados de *Staphylococcus* de pacientes internados no Complexo Hospitalar Irmandade Santa Casa de Misericórdia de Porto Alegre, com bacteremia verdadeira (invasivos) ou colonização de cateter (colonizantes). Dentre 104 *Staphylococcus* spp, 74 foram invasivos e 89% formaram biofilme, enquanto que 30 eram colonizantes de cateteres e 64% formaram biofilme ($p<0,05$). Biofilmes maduros de cinco *S. aureus* resistentes à meticilina foram expostos à vancomicina e rifampicina, e a concentração mínima para erradicar o biofilme foi 64-32.000 e 32-512 vezes maior que para células planctônicas, respectivamente. *S. aureus* susceptíveis à meticilina foram testados da mesma forma, expostos à vancomicina, eritromicina, oxacilina, gentamicina, rifampicina e tigeciclina. As melhores atividades foram encontradas para vancomicina e tigeciclina, comparado com os demais antibióticos ($p<0,05$). Dentre *S. epidermidis* formadores de biofilme, 27 foram submetidos aos mesmos testes citados acima, para diferentes antibióticos incluindo linezolida, que apresentou a melhor atividade ($p<0,05$). Finalmente, para avaliar a expressão de genes envolvidos na formação e regulação do biofilme, foi utilizada a cepa *S. epidermidis* RP62A produtora de biofilme e a técnica de qPCR. Houve um aumento de expressão dos genes *icaA*, *atlE* e *aap* após exposição à linezolida, enquanto que não houve alteração para vancomicina. Frente aos resultados, é provável que tigeciclina e linezolida possam atuar de forma mais efetiva contra biofilmes maduros formados por *Staphylococcus* spp, apesar da erradicação não ter sido evidenciada em isolados clínicos, neste estudo.

Palavras-chave: *Staphylococcus*, biofilme, resistência, expressão gênica

ABSTRACT

Staphylococcus are commensal microorganisms that inhabit the human skin, and that emerged as opportunists. *S. aureus* present remarkable virulence characteristics, causing severe diseases, whereas *S. epidermidis* is the main pathogen related to biofilm-associated infections. Biofilm are bacterial communities that survive in organized structures, and its presence in chronic infections is associated to its resistance presented against antibiotics commonly used to treat these infections. This resistance may occur mostly due to biofilm structure and fisiology. The study was performed with phenotypic methods to evaluate the biofilm formation in *Staphylococcus* spp, and with molecular tools to evaluate the expression of biofilm-associated genes. Both evaluations were based on treated and non-treated biofilms. *Staphylococcus* were selected among patients attending at Irmandade Santa Casa de Misericórdia de Porto Alegre, with true bacteraemia (invasive) or catheter colonization (colonizing). Among 104 *Staphylococcus* spp, 74 were invasive and 89% formed biofilm, whereas 30 were catheter colonizer and 64% formed biofilm ($p<0.05$). Mature biofilms of five methicillin-resistant *S. aureus* were exposed to vancomycin and rifampicin, and the minimum biofilm eradication concentration was 64-32.000 and 32-512 folds higher than for planktonic cells, respectively. Methicillin-susceptible S. aureus were tested in the same way, exposed to vancomycin, erythromycin, oxacillin, gentamicin, rifampicin and tigecycline. Superior activities were found for vancomycin and tigecycline compared with others ($p<0.05$). Among biofilm-formers *S. epidermidis*, 27 were submitted to the same tests mentioned above, including linezolid that presented better activity ($p<0.05$). Finally, the strain *S. epidermidis* RP62A was used to evaluate the expression of biofilm-associated genes using qPCR. There was an increase in the expression of *icaA*, *atlE* and *aap* after exposure to linezolid, whereas there were no alterations for vancomycin. Based on the results, it's likely that tigecycline and linezolid may act more effectively against mature biofilms formed by *Staphylococcus* spp, despite no eradication was evidenced in clinical isolates, in this study.

Key-words: *Staphylococcus,* biofilm, resistance, gene expression

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AI	Autoindutores
EPS	Extracellular Polymeric Substances
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
PIA	Polysaccharide Intercellular Adhesin
PGA	Poly-ϒ-glutamic acid
PNAG	Poly-N-acetyl-glucosamine
PSMs	Phenol-Soluble Modulins
QS	Quorum-Sensing
SCoN	*Staphylococcus* Coagulase-negativos
VISE	Vancomycin-intermediary *Staphylococcus epidermidis*

SUMÁRIO

1 INTRODUÇÃO AO GÊNERO *Staphylococcus* spp

Staphylococcus são bactérias gram-positivas que incluem diversas espécies patogênicas oportunistas, envolvidas em infecções hospitalares ou adquiridas na comunidade. Os principais representantes patogênicos são *Staphylococcus epidermidis* e *S. aureus* (Bannerman 2003; Feil *et al.* 2003). A maioria das espécies, entretanto, está primariamente associada com microbiota saprófita no ser humano.

Staphylococcus aureus é um microrganismo oportunista, responsável por uma grande variedade de infecções no ser humano, incluindo septicemia, endocardite, pneumonia e infecções de pele e ossos. Sua prevalência é alta especialmente em pacientes com fibrose cística, onde é causador de infecções respiratórias sérias (Feil *et al.* 2003). Devido ao seu potencial patogênico e a capacidade de se adaptar rapidamente à pressão seletiva dos antibióticos, pode se disseminar, sendo um patógeno emergente em várias partes do mundo (Deurenberg *et al.* 2007).

Sendo considerado primariamente colonizante da pele humana e mucosas de indivíduos saudáveis, *S. epidermidis* como comensal apresenta ampla flexibilidade em se adaptar a diferentes nichos e condições (Otto 2009). Assim como *S. aureus*, *S. epidermidis* é um dos maiores causadores de infecções associadas a internações hospitalares, principalmente quando estas envolvem o uso de dispositivos médicos como cateteres intravenosos, próteses valvares dentre outros tipos de biomateriais (Rogers, Fey e Rupp 2009). Ao contrário do *S. aureus*, *S. epidermidis* possui poucas características de virulência, causando condições patológicas menos agressivas ao paciente. Porém, infecções associadas a este patógeno são consideradas recalcitrantes à terapia antimicrobiana, tanto devido às altas taxas de resistência quanto a sua capacidade de formar biofilme em superfícies inertes de dispositivos médicos (Mack *et al.* 2006).

Colonização com *S. epidermidis* exerce um papel importante para manutenção da microbiota de pele saudável, através da competição com microrganismos potencialmente patogênicos como, em particular, *S. aureus* (Otto 2012). A maioria das doenças causadas por *S. epidermidis* e outros *Staphylococcus* coagulase-negativos (SCoN) são de caráter crônico e ocorrem como infecções relacionadas a dispositivos médicos e suas complicações (Rogers, Fey e Rupp 2009). Em infecções causadas por SCoN, remoção completa do dispositivo infectado e uso de terapia antimicrobiana prolongada são frequentemente necessários.

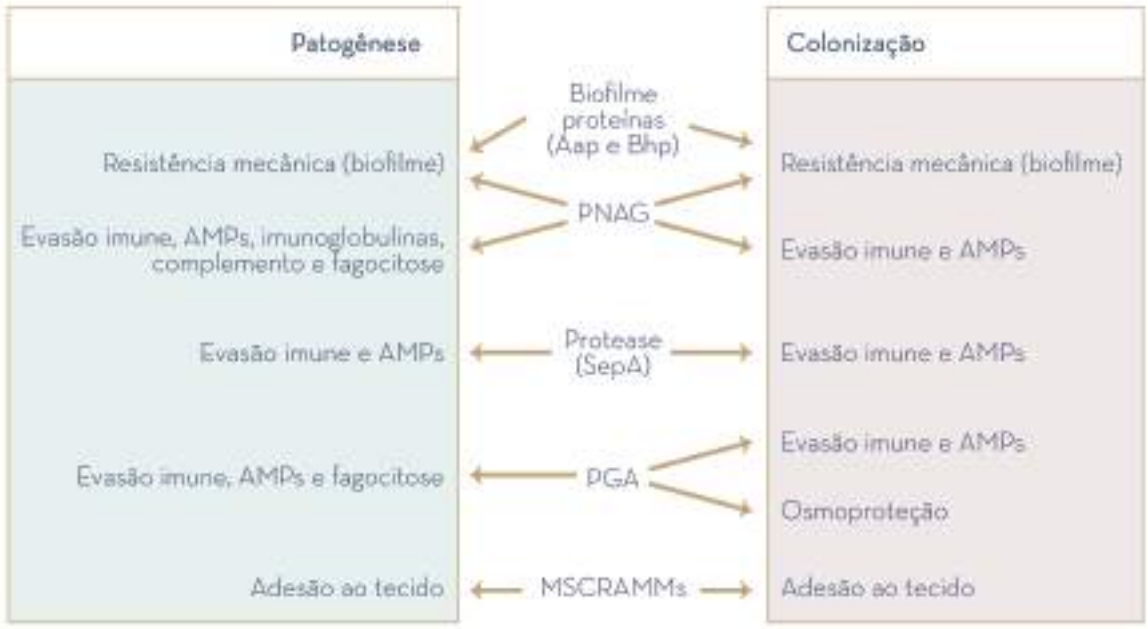
Infecções de corrente circulatória causadas por estes microrganismos são originárias de infecções associadas a cateter intravascular, e estão estimadas em 250 mil casos/ano nos Estados Unidos, representando um alto custo à saúde pública (O'Grady *et al.* 2002; Raad, Hanna e Maki 2007). As infecções por *S. epidermidis* ocorrem, principalmente, quando a integridade da pele é rompida e a bactéria frequentemente faz uso dos dispositivos médicos como cateteres intravenosos como veículos para entrar no hospedeiro (Schoenfelder *et al.* 2010).

Infecções associadas a biomateriais são complicações sérias em ortopedia, o que ultimamente tem levado à osteomielites com efeitos ósseos e em tecidos moles adjacentes (Rogers, Fey e Rupp 2009). A falha no implante é frequentemente determinada pela infecção que, uma vez tornando-se crônica, não responde mais à terapia antimicrobiana sistêmica convencional e debridamento. Sob estas circunstâncias, a substituição do implante representa a única chance real de erradicar a infecção, embora o risco de reincidência ainda seja alta (O'Grady *et al.* 2002).

Muitos estudos tem se empenhado em identificar os determinantes que diferenciam isolados de *S. epidermidis* capazes de causar infecção daqueles que vivem de maneira comensal na pele do hospedeiro. Estes estudos focam em determinantes de virulência ou usam técnicas de análise do genoma total como hibridização genômica. Dois determinantes de invasividade já foram identificados e associados a essa capacidade de causar infecção: os genes do locus *ica*, que regulam a expressão da PIA (da sigla em inglês "Polissacharide Intercellular Adhesin"), discutido a seguir; e o elemento de inserção IS256 que parece influenciar a capacidade de adaptação genética da bactéria durante a infecção. Além disso, outros fatores podem também estar associados com o equilíbrio infecção/colonização (Figura 1) (Rohde *et al.* 2004; Otto 2009).

S. epidermidis poderia ser considerado um patógeno oportunista, onde a importância clínica resulta menos de um estilo de vida infeccioso e virulento e muito mais a partir da frequência de eventos de contaminação, e a existência de mecanismos, tais como aderência e evasão imune, que são benéficos para o microrganismo tanto durante a colonização como durante uma infecção crônica (Otto 2009).

Figura 1. *S. epidermidis* como um microrganismo comensal e infeccioso.



Fonte: adaptado de Otto (2009).

Legenda: PNAG, poly-N-acetyl-glucosamine; PGA, poly-ϒ-glutamic acid; MSCRAMMs, Microbial Surface Components Recognizing Adhesive Matrix Molecules; AMP, antimicrobial peptide. Aap e Bhp são proteínas envolvidas na formação do biofilme, e SepA é uma protease que participa da evasão imune.

2 RESISTÊNCIA AOS ANTIBIÓTICOS

O principal determinante de resistência em *Staphylococcus* é a resistência aos β-lactâmicos, caracterizada laboratorialmente como resistência à meticilina. Ela é altamente frequente em hospitais entre isolados de *S. aureus* (MRSA) e SCoN, em particular *S. epidermidis* (MRSE), alcançando taxas globais de 75-90% de resistência a esse antimicrobiano (Diekema *et al.* 2001). Essa resistência ocorre devido à presença do gene *mecA*, que codifica para uma proteína ligante de penicilina com baixa afinidade aos β-lactâmicos (Chambers, Hartman e Tomasz 1985). Uma análise comparativa de três genomas de *S. aureus* revelou que, dentro de um plano genético relativamente constante, a plasticidade nas espécies é conferida por transferência horizontal de elementos genéticos grandes e de origem desconhecida, que se inserem no genoma (Ito *et al.* 2003). As cepas de MRSA, em sua

evolução e proteção contra a pressão seletiva, adquiriram e integraram ao seu genoma um elemento genético móvel de 21 a 67 kb, denominado “staphylococcal cassette chromosome” (*SCCmec*), que contém o gene de resistência à meticilina (*mecA*) e outros determinantes de resistência a antibióticos (Ma *et al.* 2002). Além disso, contém genes regulatórios (*mecI* – repressor do gene *mecA* e *mecR1* – indutor do gene *mecA*), sequência de inserção e genes que codificam para as recombinases responsáveis pela excisão e mobilidade deste elemento (Ito *et al.* 2001).

Além do gene *mecA*, o elemento genético móvel *SCCmec* é caracterizado basicamente pela presença de repetições terminais diretas e invertidas, dois elementos genéticos essenciais (complexo *mec* e complexo *ccr*) e as regiões “junkyard” (Ito *et al.* 2001; Ma *et al.* 2002). As repetições terminais possuem a propriedade de encaixe do elemento móvel no genoma bacteriano, especificado pela complementariedade de base*S.* O complexo *mec* é composto por IS431mec, *mecA* e regiões intactas ou truncadas dos genes regulatórios *mecR1* e *mecI*. O complexo *ccr* codifica para as recombinases (*ccr*) que medeiam a integração e excisão do *SCCmec* a partir do cromossomo e são, por isso, responsáveis pela mobilidade deste elemento. O restante do *SCCmec* compreende as regiões J (“junkyard”) que estão localizadas entre e ao redor dos complexos *mec* e *ccr* e contêm vários genes ou pseudogenes que não parecem ser úteis para a célula bacteriana, embora importantes exceções incluam os genes de resistência mediados por transposons ou plasmídeos para resistência aos antibióticos não β-lactâmicos e metais pesados (Ito 2003). Atualmente, existem 10 diferentes tipos de *SCCmec* que carreiam o gene *mecA* descritos para *Staphylococcus*, designados como tipos I a X, incluindo diversos subtipos, designados por letras como IVa, IVb dentre outros (http:// www.sccmec.org/Pages/SCC_TypesEN.html).

Em *S. epidermidis*, o menor elemento *SCCmec*, tipo IV, é o mais abundante (36%). Porém, ele apresenta um problema em particular, uma vez que ele não estabelece um custo de “fitness” ao seu hospedeiro, e pode por sua vez ser disseminado na ausência de antibióticos que causam pressão seletiva. Interessantemente, cepas intimamente relacionadas que carreiam diferentes tipos de *SCCmec* indicam que *S. epidermidis* frequentemente perde e adquire estes elementos genéticos móveis (Otto 2009). Um estudo realizado em 2009 em um hospital de Porto Alegre avaliou a prevalência de *SCCmec* em MRSA, e observou que o tipo mais frequentemente encontrado foi o tipo III, seguido do tipo I (Reiter *et al.* 2010).

Um novo e divergente homólogo do gene *mecA* (*mecC* ou $mecA_{LGA251}$) foi recentemente descrito em um novo *SCCmec* tipo XI (Shore *et al.* 2011). Esta proteína identificada recentemente tem <63% de identidade de aminoácidos com a PBP2a codificada pelo *mecA*, e foi descrita em *S. aureus* e SCoN. Este novo análogo do gene *mecA* já foi detectado na Inglaterra, Dinamarca e Escócia (Garcia-Alvarez *et al.* 2011).

Resistência aos aminoglicosídeos e macrolídeos e, em menor extensão, à tetraciclina, cloranfenicol e clindamicina é também frequentemente observada em isolados de *S. epidermidis* em hospitais (Rogers, Fey e Rupp 2009). Resistência intermediária à vancomicina (VISE) está surgindo (Jones *et al.* 2006), porém não existem registros de alto nível de resistência a esse antimicrobiano. Dentre os antimicrobianos mais novos, linezolida, estreptograminas, daptomicina e tigeciclina, a resistência ainda é rara, e está mais relacionada com isolados mais resistentes, como MRSE e MRSA (Rogers, Fey e Rupp 2009; Otto 2009, Dortet *et al.* 2013; Stein e Babinchak 2013).

3 FATORES DE VIRULÊNCIA

S. epidermidis é o membro mais estudado dentre os SCoN em termos de conhecimento dos seus mecanismos moleculares de virulência. Porém, comparado a *S. aureus*, sua virulência é inferior, a despeito de características superiores de multiresistência. Os fatores de virulência destes dois microrganismos podem ser caracterizados como compreendendo genes e proteínas que facilitam o estabelecimento da infecção, e persistência do organismo no corpo humano (Otto 2012).

Para *S. epidermidis*, estes fatores de virulência tem importante papel na sua vida comensal como um habitante inócuo da pele humana, e podem eventualmente não serem classificados como fatores de virulência *sensu sctricto* (Otto 2012). Porém, muitos destes fatores estão diretamente relacionados com o seu principal mecanismo de virulência, que é a capacidade de formar biofilme. Por outro lado, *S. aureus* é um microrganismo muito mais virulento, com características invasivas superiores ao *S. epidermidis.* Além disso, sua capacidade de formar biofilme está sendo mais evidenciada com os estudos nesta área, o que é agregado como mais um fator de virulência importante que faz com que este microrganismo persista em infecções crônicas (Reiter *et al.* 2012).

4 BIOFILME

Existem evidências de formação de biofilme precocemente em registros fósseis, particularmente em ambientes hidrotermais, onde microcolônias foram identificadas com a idade de 3,3-3,4 bilhões de anos em diversos lugares do planeta. Estes dados indicam que a capacidade de formar biofilme é uma característica integral e antiga dos procariotos. Neste contexto de evolução e adaptação, é provável que os biofilmes forneciam homeostase em face às condições rigorosas da terra primitiva (temperaturas e pH extremos, exposição a luz ultravioleta), facilitando assim o desenvolvimento de interações complexas entre células individuais, e fornecendo um ambiente que era suficiente para o desenvolvimento de vias de sinalização e motilidade quimiotática (Stoodley *et al.* 2002a). Além disso, facilitou interações célula-célula que requisitam proximidade, onde conseguem concentrar nutrientes (Baty *et al.* 2000).

Um biofilme é uma população ou comunidade de bactérias vivendo em organizadas estruturas em interface líquida (Davies 2003). Apesar de apresentarem várias porém similares definições, os biofilmes podem ser descritos simplesmente como um consórcio estruturado de bactérias da mesma espécie ou espécies diferentes, embebidas em uma matriz produzida por elas mesmas. Dependendo da variedade bacteriana e de condições ambientais, a matriz do biofilme irá consistir de substâncias com natureza química diversa como exopolissacarídeos, proteínas, ácidos teicóicos e DNA extracelular (eDNA) (Arciola *et al.* 2012). Microscopia confocal a laser de biofilmes formados por uma única espécie revelou que bactérias que vivem em biofilmes formam agrupamentos ou microcolônias, que são encapsuladas por uma matriz composta por diversas substâncias poliméricas extracelulares, separadas por canais de água que atuam como um sistema circulatório primitivo para entrega de nutrientes e remoção de lixo metabólico (Lawrence *et al.* 1991; Lawrence e Neu 1999).

Dentre os agentes etiológicos de infecções relacionadas ao uso de dispositivos médicos dentro do gênero *Staphylococcus*, estão *S. aureus* e *S. epidermidis* ocupando as primeiras posições, seguidos de outros SCoN emergentes como *S. hominis* e *S. haemolyticus* (Arciola *et al.* 2005; von Eiff *et al.* 2006; Campoccia *et al.* 2010). Estes microrganismos podem ser introduzidos durante a implantação do dispositivo médico ou advindos de uma bacteremia temporária. Assim, eles aderem ao biomaterial e crescem na forma de biofilme.

Após alguns estudos, o mecanismo de formação do biofilme em *Staphylococcus* começou a ser desvendado. Porém, é um mecanismo complexo e que envolve inúmeras proteínas, algumas ainda sem função específica, e que está ainda sob investigação. Baseado em modelos experimentais *in vitro*, a formação do biofilme pode ser classificada em 4 etapas distintas: 1) Adesão inicial das bactérias; 2) Agregação intercelular e acúmulo em camadas celulares; 3) Maturação e 4) Destacamento ou dispersão das células do biofilme ao seu estado planctônico inicial (Figura 2) (Mack *et al.* 2004; Costerton, Montanaro e Arciola 2005).

Figura 2. O ciclo de formação do biofilme: etapas e determinantes envolvidos.

Fonte: adaptado de Arciola *et al.* (2012).

Legenda: EPS, extracellular polymeric substances; eDNA, DNA extracelular bacteriano; PIA, polyssacharide intercelullar adhesine; AIP, autoinducer peptide. Fatores como σ^B e SarA participam da regulação da formação do biofilme, e RNAIII da dispersão celular.

4.1 ADESÃO PRIMÁRIA

Durante a primeira etapa, as interações iniciais ocorrem entre a bactéria e a superfície do biomaterial. Estas interações são inespecíficas e guiadas pela hidrofobicidade, forças eletrostáticas e de van der Waals, dentre outras. Nesta fase, as bactérias são passivamente adsorvidas à superfície abiótica do material. Neste momento, o grau de hidrofobicidade da superfície bacteriana e do biomaterial são considerados altamente importantes para a aderência inicial (Legeay, Poncin-Epaillard e Arciola 2006). Além disso, proteínas específicas tem sido identificadas como mediadoras da ligação bacteriana a superfícies abióticas (Heilmann *et al.* 1997). Autolisinas associam-se à superfície por interações hidrofóbicas ou iônicas, possuindo dupla função: enzimática e adesiva. Heilmann *et al.* (1997) demonstraram a presença de uma nova função da já conhecida autolisina AtlE em *S. epidermidis*, a qual mostrou mediar a adesão de células bacterianas ao poliestireno, representando também um pré-requisito para a formação do biofilme. Em *S. aureus*, a autolisina AtlA é altamente homóloga à AtlE, possuindo provavelmente a mesma função (Foster 2005).

4.2 AGREGAÇÃO INTERCELULAR

Durante a segunda etapa, as principais proteínas envolvidas são as chamadas MSCRAMMs (da sigla em inglês “Microbial Surface Components Recognizing Adhesive Matrix Molecules”), na qual o acúmulo de camadas celulares bacterianas ocorre através de um processo ativo e por adesão intercelular (Patti *et al.* 1994; Speziale *et al.* 2009). Durante essa fase, o biofilme é progressivamente estabelecido na superfície colonizada formando microcolônias. Logo após esta etapa inicia-se a maturação do biofilme, o qual adquire sua forma e características estruturais específicas.

4.3 MATURAÇÃO

O desenvolvimento da arquitetura peculiar do biofilme ocorre nesta terceira fase. Durante a maturação do biofilme, ocorre a expressão das mais diversas proteínas e principalmente formação da matriz, produzida pelas próprias bactérias em biofilme. É neste momento que ocorre o acúmulo de EPS (da sigla em inglês “Extracellular Polymeric

Substances"), as quais serão então os principais componentes da matriz do biofilme (Arciola *et al.* 2012).

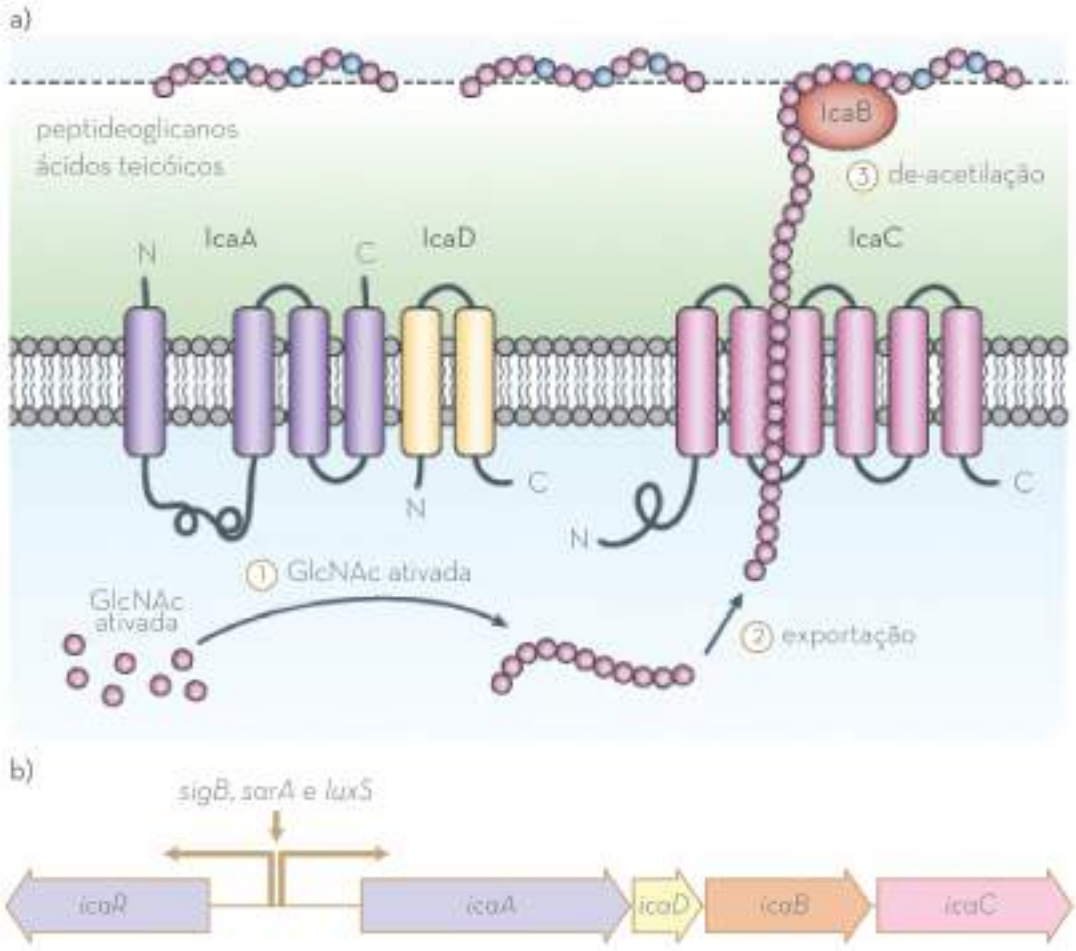
A maturação do biofilme em *Staphylococcus* pode ocorrer de duas maneiras distintas, descritas a seguir. Embora múltiplos fatores externos e bacterianos influenciam as fases iniciais de adesão e o acúmulo, a maturação é altamente influenciada pela produção de proteínas e polissacarídeos bacterianos (Arciola *et al.* 2012).

4.3.1 Maturação *ica*-dependente

A adesão célula-célula para criação de uma estrutura tridimensional característica do biofilme pode ocorrer devido à produção da adesina intercelular polissacarídica, PIA. Este polissacarídeo consiste de um glucosaminoglicano linear cuja síntese é mediada pelo lócus intercelular de adesão (*icaADBC*). Este lócus *ica* foi inicialmente descoberto e investigado em *S. epidermidis* (Heilmann *et al.* 1996) e por um longo tempo foi considerado como determinante de virulência de *S. epidermdis* envolvidos em infecções relacionadas ao uso de cateteres. Em 1999, Cramton *et al.* descobriram que o lócus ica estava presente também em *S. aureus*, assim como em outras espécies do gênero. Em *S. epidermidis*, o lócus *ica* parece ter um importante papel na patogênese de infecções relacionadas ao uso de dispositivos médicos, como demonstrado por um estudo in vitro que evidenciou a presença da PIA na evasão imune e virulência em *S. epidermidis* (Vuong *et al.* 2004a).

O produto do gene *icaA* é uma proteína transmembrana com homologia a N-acetil-glucosaminiltransferases, necessitando o produto do gene *icaD* para atividade ótima. Os oligômeros N-acetil-glucosamina produzidos pelos genes *icaAD* atingem um máximo comprimento de 20 resíduos e somente quando ocorre a coexpressão do *icaC* que cadeias maiores são sintetizadas (Gerke *et al.* 1998). IcaC também está provavelmente envolvido na translocação do polissacarídeo em extensão até a superfície celular. A proteína IcaB é ligada à superfície e por isso responsável pela deacetilação da molécula de N-acetilglucosamina (Vuong *et al.* 2004a). Se não ocorrer essa deacetilação, a bactéria não é capaz de mediar o desenvolvimento do biofilme (Figura 3).

Figura 3. A síntese do exopolissacarídeo poli-N-acetilglucosamina.



Fonte: adaptado de Otto (2009).

O gene *icaR*, transcrito divergentemente e localizado “upstream” ao lócus *icaADBC*, em *S. aureus* e *S. epidermidis*, é um regulador transcricional que codifica um repressor com papel central na regulação ambiental da expressão do lócus *ica* em *S. epidermidis* (Conlon, Humphreys e O’Gara 2002). Além do gene *icaR*, outros genes também estão envolvidos na regulação da expressão do lócus *ica*, discutidos a seguir.

4.3.2 Maturação *ica*-independente

Por um longo tempo, pensou-se que o lócus ica era estritamente necessário para formação do biofilme em *Staphylococcus.* De certa maneira, em algumas cepas de *S. aureus* que carreiam este lócus, a sua deleção não prejudica a capacidade de produzir biofilme, o qual

pode estar sendo produzido por uma via independente (O'Gara 2007). Apesar da importância inegável do lócus *icaADBC* e as vias regulatórias que controlam a produção da PIA no desenvolvimento do biofilme em *Staphylococcus*, estudos nesta década tem demonstrado a existência de um mecanismo independente de *ica* para *S. aureus* e *S. epidermidis* (Beenken *et al.* 2004; Fitzpatrick, Humphreys e O'Gara 2005; Rohde *et al.* 2007).

Um dos principais mecanismos *ica*-independente que participam da fase de maturação do biofilme em *S. epidermidis* é a proteína associada ao acúmulo Aap (da sigla em inglês "accumulation-associated protein"). A proteína Aap foi originalmente identificada por Hussain *et al.* (1997), que utilizou mutagênese para gerar um mutante sem uma proteína de 140 kDa requerida para acúmulo do biofilme. A observação de anticorpos anti-Aap inibia o biofilme ica-dependente, sugerindo que de alguma maneira a proteína Aap poderia ancorar a PIA à superfície celular. Porém, Rohde *et al.* (2005) demonstrou que a clivagem proteolítica da Aap por proteases do hospedeiro ou do microrganismo, que converte a proteína em adesina, é suficiente para mediar a maturação do biofilme de maneira *ica*-independente.

Em *S. aureus*, uma proteína homóloga à Aap é a proteína de superfície SasG, conhecida por mediar a ligação do microrganismo à superfície das células epiteliais nasais (Roche, Meehan e Foster 2003). Kuroda *et al.* (2008) já havia demonstrado que esta proteína contribuía para a autoagregação intercelular, o que facilitaria a aderência a tecidos do hospedeiro durante uma infecção por *S. aureus.* Mas, o papel desta proteína na fase de acumulação e maturação do biofilme em *S. aureus* só foi demonstrado por Geoghegan *et al.* (2010). Semelhante à Aap, esta proteína possui um domínio A N-terminal, e domínios B repetidos, os quais são clivados espontaneamente a peptídeos de ligação muito lábeis, que se mantém ligados à célula e são necessários para a formação do biofilme (Geoghegan *et al.* 2010).

4.4 DISPERSÃO

A quarta etapa da formação do biofilme é, por se dizer, a dispersão do biofilme (Figura 4). As bactérias previamente aderidas, embebidas e protegidas pela matriz e estrutura em si retornam a sua fase planctônica de vida, destacando-se do biofilme. A fase planctônica é a fase de vida livre, ou seja, não em biofilme, onde elas estão prontas para uma nova fase invasiva (Arciola *et al.* 2012). O destacamento e dispersão das células podem ser causados

por perturbações externas, como aumento da fluidez, por processos internos do biofilme, como degradação enzimática endógena, ou por liberação do EPS ou proteínas de superfície (Boyd e Chakrabarty 1994; Stoodley *et al.* 2002b, Kaplan *et al.* 2003). Em algumas espécies, a dispersão pode ser um processo ativo, presumidamente adaptado para permitir a colonização de novos nichos (Sauer *et al.* 2005).

Figura 4. Modelo das formas de dispersão do biofilme em *Staphylococcus.*

Fonte: adaptado de Boles e Horswill (2011).

Três estratégias distintas de dispersão do biofilme podem ser identificadas: 1) dispersão por semeadura (“swarming/seeding”), onde células individuais são liberadas da microcolônia em direção ao exterior do biofilme; 2) dispersão por aglutinação (“clumping”), onde agregados celulares são lançados como êmbolos; e 3) dispersão por superfície (“surface”), onde estruturas do biofilme movem através de superfícies (Hall-Stoodley, Costerton e Stoodley 2004).

S. aureus e *S. epidermidis* utilizam um mecanismo principal para dispersão das células em biofilme que se baseia na produção de enzimas extracelulares e surfactantes, que degradam e solubilizam componentes adesivos da matriz do biofilme. Devido a esta matriz embeber as células bacterianas dentro de uma microcolônia, a degradação da matriz resulta no

destacamento de células da microcolônia e a liberação destas no ambiente. Os produtos gênicos implicados na degradação de componentes da matriz incluem proteases, DNases e surfactantes, e o sistema regulatório que controla essa produção é o sistema *agr* (da sigla em inglês “accessory gene regulator”), discutido a seguir (Boles e Horswill 2011).

Além de complexo, o mecanismo de dispersão do biofilme em relação ao tempo e extensão dessa dispersão depende de diversas condições ambientais. Sob condições favoráveis, a maioria dos biofilmes do tipo selvagens, ou seja, livres de mutações, liberam um pequeno número de células de forma contínua, mas também sofrem um destacamento esporadicamente maior e por um período maior de tempo (Yarwood e Schlievert 2003). Um exemplo de evento que promove um destacamento maior de células, em *S. aureus*, é a remoção de glicose do meio de cultura. Em condições normais, a glicose reprime o sistema *agr*; porém quando ausente do ambiente, este sistema é ativado e a produção de enzimas que degradam a matriz é iniciada (Boles e Horswill 2008). Outras mudanças ambientais provavelmente podem alterar as condições fisiológicas do biofilme, tanto promovendo a formação quanto a dispersão deste (Boles e Horswill 2011).

4.5 QUORUM-SENSING

Um importante componente em biofilmes bacterianos é a capacidade de comunicação utilizando os sistemas quorum-sensing (QS). Os sinais destes sistemas são pequenas moléculas chamadas autoindutores (AIs). Em uma baixa densidade de população celular, AIs estão em baixas concentrações. Quando as células atingem certa densidade populacional, os AIs acumulam em um limiar de concentração que ativa o regulador transcricional. Este, por sua vez, regula a expressão de vários genes, os quais frequentemente incluem uma série de fatores de virulência (Kong, Vuong e Otto 2006; Novick e Geisinger 2008; Boles e Horswill 2011).

O sistema mais importante em *Staphylococcus* é o sistema *agr*, que não parece afetar a expressão do lócus *ica* e produção de PIA. Um segundo sistema QS descrito para estes microrganismos é o sistema LuxS, recentemente emergido como outro regulador negativo da formação de biofilme em *S. epidermidis* (Xu *et al.* 2006). Por fim, existe também uma regulação que envolve a sinalização de estresses ambientais, promovida pelos fatores σ. O regulador da resposta global ao estresse σB e a proteína RsbU (regulador positivo do σB)

controlam o desenvolvimento do biofilme em *S. epidermidis*, mas não em *S. aureus* (O'Gara 2007).

4.5.1 Sistema Agr

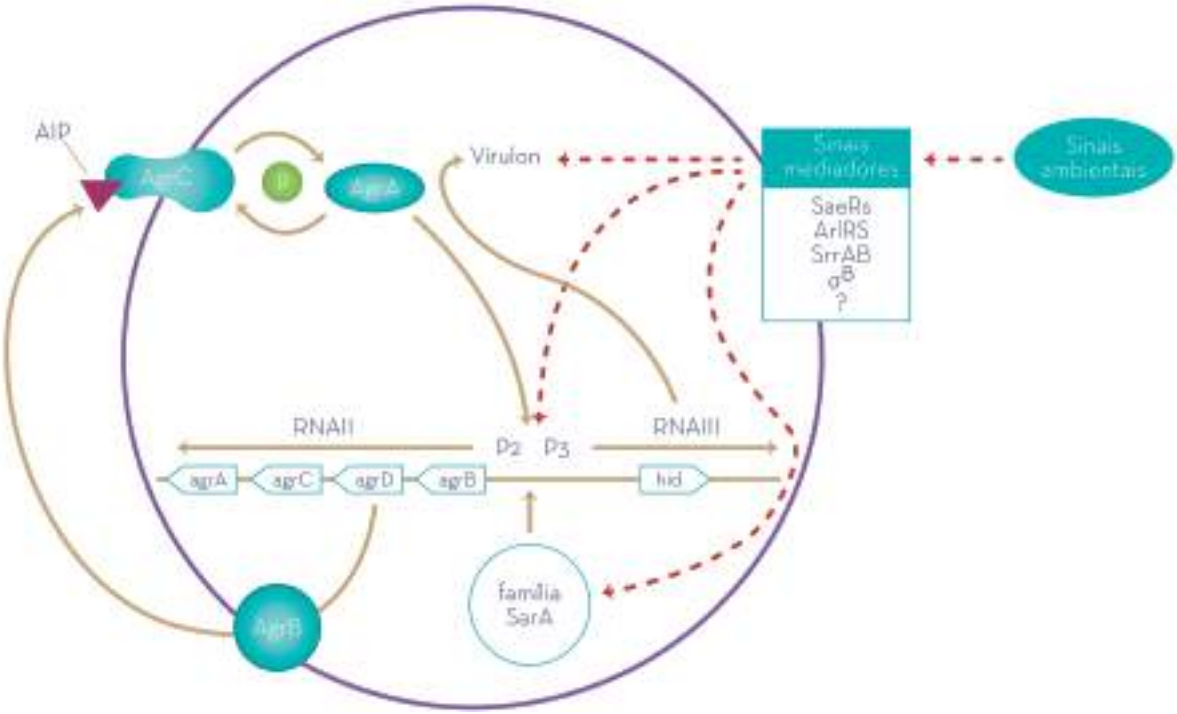
O sistema Agr é composto por duas unidades transcritas divergentemente, RNAII e RNAIII, as quais são transcritas por dois promotores P2 e P3, respectivamente. A unidade RNAII contém 4 genes, *agrB*, *agrD*, *agrC* e *agrA* (Novick *et al.* 1995). Os produtos dos genes *agrB* e *agrD* estão ligados à produção de AIs, que se ligam à proteína transmembrana AgrC, a qual age como um sensor quinase deste sistema. Assim que ocorre a ligação, AgrC ativa o regulador de resposta AgrA, que induz a transcrição de RNAII e RNAIII (Figura 5) (Kong, Vuong e Otto 2006; Novick e Geisinger 2008). Quando o sistema *agr* está não funcional, ocorre uma facilitação da adesão inicial das células bacterianas à superfície abiótica como poliestireno, presumariamente pela regulação positiva da adesão e regulação negativa de moléculas de dispersão (Vuong *et al.* 2000). Por exemplo, a proteína AtlE, é super expressa em *S. epidermidis* mutantes do lócus agr.

Uma vez aderida, a bactéria começa a fase de acúmulo em massa, construindo a arquitetura celular com múltiplas camadas. Esta adesão célula-célula e consequente maturação do biofilme é considerada determinante e envolve principalmente a produção da PIA (Heilmann *et al.* 1997). Porém, sua produção não é regulada pelo sistema *agr*, mas sim pelo outro sistema QS LuxS, o qual diminui a produção deste polissacarídeo (Xu *et al.* 2006).

Após a maturação do biofilme, pode haver o destacamento de pequenos grupos celulares que dispersam do biofilme maduro. Estes grupos podem disseminar a locais distantes. Assim, a dispersão do biofilme pode ser muito importante para persistência da infecção associada à formação de biofilme (Kong, Vuong e Otto 2006). O sistema *agr* controla este processo de dispersão do biofilme, uma vez que cepas mutantes deste sistema apresentam um biofilme mais denso do que cepas selvagens (Vuong *et al.* 2000; Yarwood, Volper e Greenberg 2005; Yao, Sturdevant e Otto 2005). Este aumento na densidade do biofilme não é atribuído ao crescimento celular ou morte, mas sim à incapacidade das células se destacarem do biofilme maduro (Vuong *et al.* 2004b). Um estudo por microscopia confocal demonstrou que a dispersão de células do biofilme coincide com a expressão do sistema *agr* (Yarwood e Schlievert 2003). A falha no deslocamento de células do biofilme é

provavelmente associado à ausência de produção de pequenos peptídeos nos mutantes, chamados PSMs (da sigla em inglês “phenol-soluble modulins”). *S. aureus* e *S. epidermidis* possuem um grupo distinto de PSMs que inclui a delta-toxina codificada pelo gene *hld* contido na unidade RNAIII. Provavelmente devido à natureza anfifática é que estes peptídeos promovem o destacamento de células bacterianas do biofilme (Kong, Vuong e Otto 2006).

Figura 5. O sistema Agr em *Staphylococcus.*



Fonte: adaptado de Yarwood e Schlievert (2003).

Uma proteína de regulação global associada com o sistema *agr* é a SarA, que pode ter função de ativar ou reprimir a transcrição de genes. Esta proteína afeta e expressão de genes de virulência em *S. aureus* e *S. epidermidis*, e o *agr* é regulado positivamente. Devido ao fato de que SarA é um ativador do *agr*, que por sua vez reprime a formação do biofilme, pode se dizer que uma mutação nesse gene aumenta a capacidade de formação de biofilme (Cue, Lei e Lee 2012).

4.5.2 Sistema LuxS

O sistema QS LuxS é presente em uma grande variedade de microrganismos gram-positivos e gram-negativos. A função bioquímica da proteína LuxS em *S. aureus* é produzir um autoindutor AI-2, e ocorre de maneira muito similar em *S. epidermdis*. A forma de regulação da formação do biofilme mediada por este sistema é semelhante ao sistema *agr*, uma vez que a cepa mutante também forma um biofilme mais denso e mais compacto comparado à cepa selvagem. Porém, em contraste à molécula efetora da via no sistema *agr*, a delta-toxina, que promove a dispersão de células do biofilme, *luxS* parece influenciar a formação do biofilme através da regulação transcricional do lócus *ica*, consequentemente alterando a produção de PIA (Kong, Vuong e Otto 2006; Cue, Lei e Lee 2012). Em *S. epidermidis*, o aumento da formação do biofilme está correlacionada com expressão reduzida de *luxS* e aumento da produção de PIA. Em ambos sistemas, o biofilme se torna mais denso nas cepas mutantes, porém por mecanismos distintos em cada um dos sistemas QS (Cue, Lei e Lee 2012).

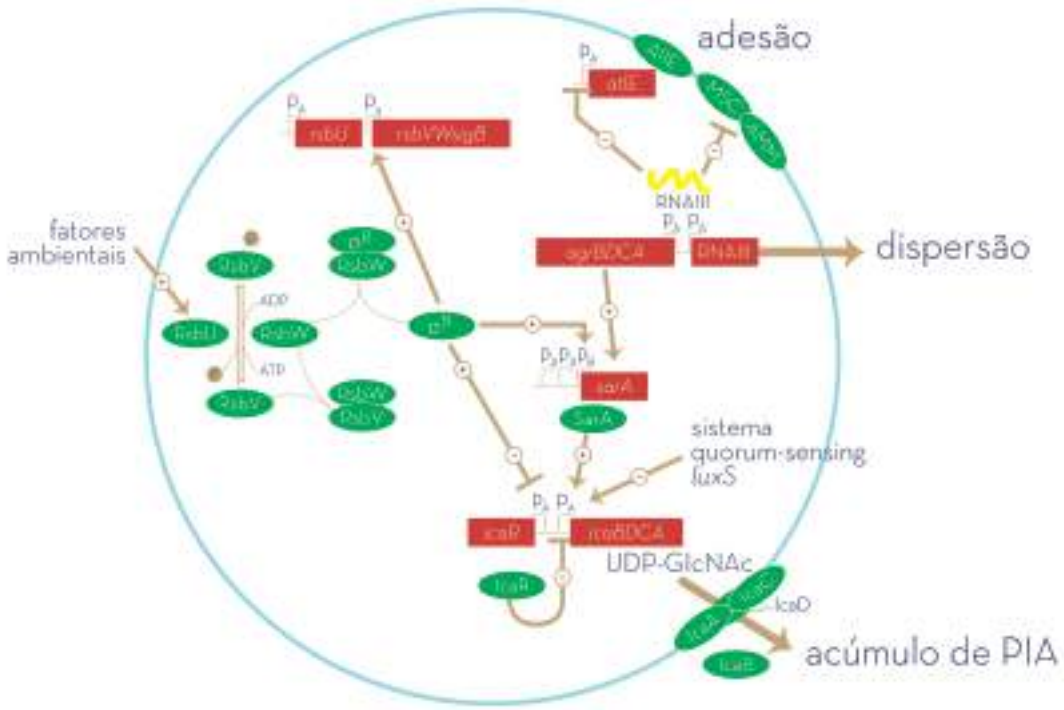
4.5.3 Regulação ambiental

SigmaB é um fator σ alternativo encontrado em *Staphylococcus* que é responsável pela resposta ao estresse causado pelo ambiente onde se encontra o microrganismo (Conlon, Humphreys e O'Gara 2004). Ele é ativado por sinais como alta temperatura, alta osmolaridade, antibióticos e pH extremo. A transcrição do lócus *sigB* é guiado por 3 promotores distintos. O primeiro produz um transcrito que codifica para *rsbUVW* e *sigB*, e o segundo guia a síntese de um transcrito menor sem *rsbU* (Cue, Lei e Lee 2012).

A atividade do σB é controlada por diversas quinases e fosfatases. Na ausência de estresse, o σB está inativado pois associa-se ao fator anti-σ, RsbW. Ele também fosforila e inativa o fator anti-anti-σ RsbV. Quando existe estresse, a enzima fosfatase RsbU desfosforila o RsbV, que então dissocia o σB do fator anti-σ. O σB liberado pode então se associar à RNA polimerase. No geral, σB pode promover a formação do biofilme por reprimir a produção de proteases e toxinas, um efeito que é manifestado através da diminuição da expressão de RNAIII bem como uma possível ativação de *sarA*. Além disso, σB pode também ativar a formação de biofilme *ica*-independente (Arciola *et al.* 2012; Cue, Lei e Lee 2012). Um

esquema envolvendo uma pequena parte do mecanismo regulatório do biofilme pode ser observado na Figura 6 (Mack *et al.* 2007).

Figura 6. Modelo da regulação da formação do biofilme em *Staphylococcus.*



Fonte: adaptado de Mack *et al.* (2007).

4.6 BIOFILME E RESISTÊNCIA

Biofilmes bacterianos são resistentes a antibióticos, desinfetantes, fagocitose e outros componentes da defesa imune inata e adaptativa do hospedeiro (Costerton, Stewart e Greenberg 1999; Stewart 2002). Nos anos 90, a importância do biofilme foi destacada por Costerton, Stewart e Greenberg (1999), que apontaram que a formação de comunidades bacterianas em biofilme e sua resistência inerente aos antibióticos eram a raiz de muitas infecções crônicas em persistentes. Graças a compacta natureza da estrutura do biofilme, as presumidas taxas menores de crescimento celular e respiração das células em biofilme e a proteção conferida pela matriz, agentes químicos naturais e artificiais são incapazes de atacar e destruir adequadamente as populações em biofilme (Passerini *et al.* 1992; Pople, Bayston e

Hayward 1992). O aumento da resistência aos antibióticos é muito associado com bactérias em biofilme. Quando aderidas, as bactérias apresentam uma resistência 10-1000 vezes superior a diversos agentes antimicrobianos do que a mesma bactéria em sua fase de crescimento planctônico (Davies 2003).

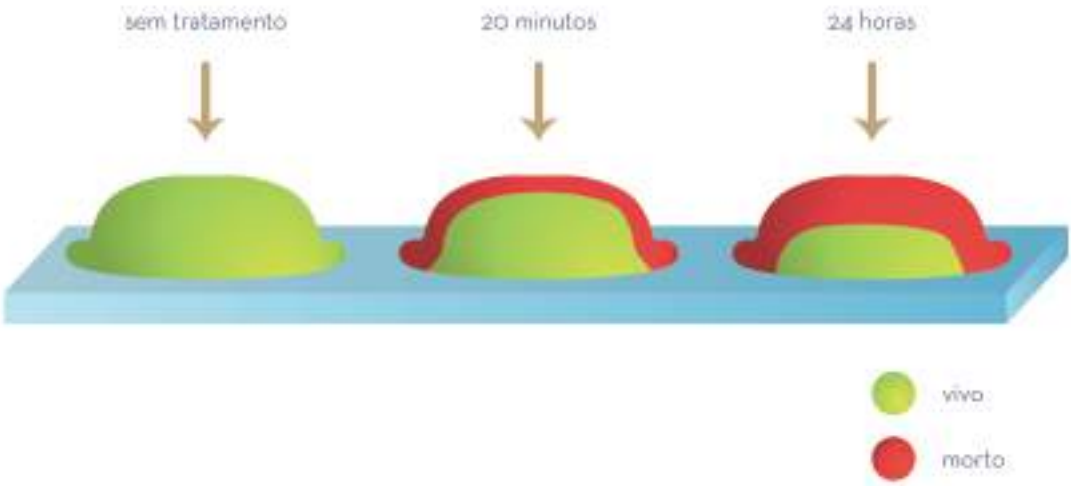
Uma análise estrutural do biofilme mostra que cerca de 15% em volume é constituído por células bacterianas, embebidas em uma matriz na qual canais carreiam líquido para dentro da comunidade bacteriana em um fluxo convectivo. A diferenciação fisiológica de células sésseis *versus* células planctônicas, bem como a complexidade da estrutura do biofilme, sugere que comunidades bacterianas que formam biofilme são finamente organizadas e necessariamente reguladas por sinais análogos a hormônios e ferormônios típicos de comunidades multicelulares de células eucarióticas (Davies *et al.* 1998).

Três mecanismos principais foram propostos para explicar a resistência dos biofilmes a agentes antibióticos. O primeiro é baseado nas propriedades de barreira da matriz. Este mecanismo pode ser mais relevante para agentes antimicrobianos reativos (superóxidos), carregados (metais) ou grandes (imunoglobulinas) que são neutralizados ou ligados ao EPS e eficientemente diluídos a concentrações sub-letais antes que eles possam atingir as células bacterianas individuais no biofilme. Essa propriedade de barreira também pode localizar e concentrar atividades enzimáticas, como por exemplo β-lactamases que degradam antibióticos β-lactâmicos (Hall-Stoodley, Costerton e Stoodley 2004) e expressar fatores de proteção específicos como bombas de efluxo que ativamente impedem o acesso do antibiótico à célula. Além disso, pode ocorrer um impedimento estérico devido às características da matriz, dificultando ou até mesmo bloqueando a penetração do antibiótico no biofilme (Davies 2003).

O segundo mecanismo envolve o estado fisiológico do biofilme. Embora muitos antibióticos consigam penetrar livremente no EPS, células dentro do biofilme estão frequentemente protegidas. A reduzida taxa de crescimento de células em biofilme gera a criação de fases dormentes e zonas estacionárias no biofilme, o que parece ser um fator significante para a resistência aos antibióticos (Spoering e Lewis 2001; Anderl *et al.* 2003; Davies 2003; Walter *et al.* 2003). Isso ocorre particularmente para antibióticos como β-lactâmicos e glicopeptídeos, que são efetivos contra *Staphylococcus* em divisão ativa, pois interrompem a síntese da parede celular. Porém, estes antibióticos requerem um mínimo grau de atividade celular para serem efetivos, devido ao seu mecanismo de ação envolver o rompimento do processo de divisão celular. A Figura 7 representa a atividade dos antibióticos

contra uma população em biofilme típica. O tratamento inicial é geralmente efetivo somente para células marginais das microcolônias, ou seja, aquelas que estão localizadas mais próximas ao meio exterior. Por outro lado, as bactérias localizadas nas camadas mais profundas destas microcolônias não são sempre atingidas e podem potencialmente sobreviver, continuando a disseminação da infecção (Davies 2003).

Figura 7. Comportamento do biofilme após exposição a antibióticos.



Fonte: adaptado de Davies (2003).

O terceiro mecanismo é a existência de subpopulações com fenótipos resistentes, os quais são referidos como “persisters” - células persistentes (Spoering e Lewis 2001). Estas células compreendem uma fração pequena da biomassa completa, sendo uma cultura planctônica ou séssil. Após a eliminação de possíveis células sensíveis aos antibióticos, as células persistentes permanecem e podem repovoar o biofilme (Hall-Stoodley, Costerton e Stoodley 2004). Shapiro, Nguyen e Chamberlain (2010) conduziram um estudo para determinar se a cepa *S. epidermidis* RP62A (ATCC 35984, produtora de biofilme) poderia sobreviver como célula persistente em culturas planctônicas e em biofilme, após tratamento com antibióticos como levofloxacina e vancomicina. Neste estudo, a forma planctônica mostrou menos de 1% de células persistentes e a cultura séssil mostrou 28 e 94% após tratamento com levofloxacina e vancomicina, respectivamente.

Muitos investigadores mostram que biofilmes têm taxas de crescimento reduzidas e que isso é o principal fator da resistência a antibióticos clinicamente efetivos para tratamento. O grupo de pesquisa Soren Molin da Universidade Técnica da Dinamarca é um dos líderes no estudo da fisiologia do biofilme, e verificou através de marcadores metabólicos marcados com fluorescência que, de fato, os centros das microcolônias dos biofilmes possuem taxas metabólicas reduzidas comparadas com as células mais próximas da superfície (Davies 2003).

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6 OBJETIVOS

Este estudo foi desenvolvido utilizando isolados bacterianos de *Staphylococcus* spp provenientes de pacientes internados no Complexo Hospitalar Irmandade Santa Casa de Misericórdia de Porto Alegre (CHISCPA). Estes isolados foram selecionados a partir de hemoculturas e pontas de cateter.

Objetivos específicos:

1) Comparar *Staphylococcus* spp de hemoculturas e pontas de cateter de acordo com características clínicas e microbiológicas, segregados nos grupos ‘invasivo’ e ‘colonizante’;
2) Verificar a prevalência da formação de biofilme;
3) Verificar a prevalência do principal determinante de resistência, gene *mecA*, e os tipos de *SCCmec* associados a isso;
4) Determinar as concentrações inibitórias mínimas (CIM) para diferentes antibióticos: vancomicina, eritromicina, gentamicina, tigeciclina, oxacilina, rifampicina e linezolida;
5) Determinar as concentrações inibitórias mínimas (CMIB) e as concentrações para erradicar o biofilme (CMEB) já formado para os mesmos antibióticos já citados;
6) Comparar a CIM com a CMIB e CMEB para cada antibiótico e cada isolado;
7) Determinar a prevalência de genes associados à formação do biofilme em *S. aureus*, *atlA*, *icaA* e *sasG*.
8) Verificar a expressão de genes associados à formação e regulação do biofilme (*icaA*, *atlE*, *aap*, *rnaIII*, *icaR*, *luxS*, *sarA* e *rsbU*) antes e após a exposição a antibióticos utilizados na clínica, contra biofilmes maduros da cepa padrão *S. epidermidis* RP62A (vancomicina e linezolida);
9) Verificar a densidade dos biofilmes maduros após a exposição à vancomicina e linezolida, ao longo do tempo e com diferentes concentrações.

7 PRODUÇÃO CIENTÍFICA

7.1 CAPÍTULO 1

High biofilm production by invasive multiresistant staphylococci

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High biofilm production by invasive multiresistant staphylococci

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Biofilm-forming staphylococci are known for being opportunistic and invasive pathogens that cause severe disease, mostly catheter-related infections. Early detection and pathogenic strains carrying highly transferable resistance cassettes epidemiology are essential for infection spread control. Hence, this study was designed to evaluate staphylococci biofilm formation and SCCmec typing. Biofilm production and SCCmec typing were evaluated using a semi-quantitative method based on microtiter plates and a multiplex PCR for types, I–V, respectively. Blood cultures and peripheral intravenous device (IVD) staphylococci were consecutively enrolled and allocated into two different groups (invasive and colonizing) based on clinical and microbiological criteria. Seventy-four invasive and 30 colonizing isolates from distinct patients were studied. Vancomycin was the most administrated antimicrobial agent among these patient's treatments. Biofilm formation was observed in 89% of invasive and 64% of colonizing isolates ($p < 0.05$). There was significant difference regarding SCCmec typing between colonizing and invasive isolates when harboring SCCmec types IV or V ($p < 0.05$), but no correlation between biofilm intensity and SCCmec types was verified. The SCCmec elements spread are still ongoing and for that reason, antimicrobial resistance evolution in invasive and colonizing biofilm-forming staphylococci is highly relevant.

Key words: *Staphylococcus*; *Staphylococcus aureus*; biofilm; SCCmec.

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Biotic or abiotic surface adherence is the critical first event in staphylococci infection establishment (1). Skin staphylococci are known for being opportunistic pathogens and very frequently infecting catheter materials (2, 3). *Staphylococcus aureus* harbors wide adhesins variety that mediates attachment to host factors, such as extracellular matrix and plasma proteins (1). It has been shown that especially *S. epidermidis* cause a new infection that is described as 'chronic polymer-associated infection'. Colonized devices may become bloodstream infection primary focus and invasive pathogens could cause severe infections, like bacteremia under favorable conditions (4).

Biofilms are formed basically by two different stages: primary bacteria attachment onto polymer surfaces and further cell-cell proliferation to form multilayered clusters (5). Thus, in catheter-related infections, antimicrobial therapy is nearly impossible, because antimicrobials can barely penetrate the slime capsule of

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biofilm-forming bacteria (6). As consequence, such infections contribute to persistence, multiresistance, and maybe, to invasiveness of biofilm-forming bacteria in the human body.

Staphylococci biofilm-forming capacity contributes to antimicrobial resistance, otherwise is contradictory (7–9). *Staphylococcus* resistance to β-lactamic antimicrobials is mediated by a modified penicillin-binding protein (PBP2a), encoded by mecA gene (10). This gene is inserted in a mobile genetic element called staphylococcal cassette chromosome mec (SCCmec) (11) that has several major variants distinguished. Traditionally, SCCmec types I, II, and III are multiresistant hospital-acquired cassettes, and SCCmec types IV and V carry only mecA gene and are community-associated cassettes (12). The SCCmec structure and distribution in *S. aureus* and coagulase-negative *Staphylococcus* (CoNS) have been studied frequently (13–16) and it could be an important factor for multiresistance development (16, 17). Besides, SCCmec is a vehicle for exchanging resistance genes between staphylococci due to ccr complex (11, 14), and biofilm formation might be a substantial advantage for this process.

As biofilm production could be a virulence marker associated to multiresistance, this study was designed to evaluate biofilm formation and SCCmec typing using staphylococci isolates from patients attending at a tertiary hospital care.

MATERIALS AND METHODS

Setting and bacterial isolates

Staphylococci strains from patients attending at Complexo Hospitalar da Santa Casa de Porto Alegre (Porto Alegre, Brazil) were analysed.

One hundred and four consecutive staphylococcal strains were selected from distinct patients and allocated into two different groups based on clinical and microbiological criteria: invasive and colonizing strains.

Staphylococcal strains isolated from two consecutive 3-day-interval paired blood cultures of patients with temperature >38 °C, chills and septic appearance were considered invasive strains. Staphylococcal strains from peripheral IVD of patients with no fever, no septic appearance, and whose peripheral blood culture was negative for staphylococci were considered colonizing strains. Antimicrobial therapy and treatment period were recorded.

Phenotypic identification at species level was performed as described previously (18). Antimicrobial susceptibility test for gentamicin (10 μg), doxycycline (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), clindamycin (2 μg), sulfametoxazole-trimethoprim (25 μg), and cefoxitin (30 μg) (Oxoid, Cambridge, UK) was performed by disk diffusion according CLSI (19). Microdilution method was used for vancomycin (lot. no 088K06811; Sigma-Aldrich, St. Louis, MO, USA) (19).

SCCmec molecular typing

Gene mecA presence was performed as described elsewhere (20), and SCCmec typing was determined as described by Zhang et al. (21), with some modifications. Briefly, 1 μL of bacterial DNA was added to 24 μL of PCR mixture containing Tris–HCl buffer (pH 8.4), 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate (Invitrogen Inc., Carlsbad CA, USA), various concentrations of the respective primers I, II, III, IVa, IVb, IVc, IVd, and V (data not shown), and 1.25 unit of Platinum Taq DNA polymerase (Invitrogen Inc., Carlsbad, CA, USA).

Amplification was performed in a LifePro Thermal Cycler (Hangzhou Bioer Technology Co. Ltda, Hangzhou, China) beginning with an initial denaturation step at 94 °C for 5 min followed by 10 cycles of 94 °C for 45 s, 63 °C for 45 s, and 72 °C for 1.5 min and another 25 cycles of 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 1.5 min, ending with a final extension step at 72 °C for 10 min.

The SCCmec typing standard control strains, including type I (NCTC10442), type II (N315), type III (85/2082), type IVa (CA05), type IVb (8/6-3P), type IVc (MR108), type IVd (JCSC4469), and type V (WIS [WBG8318]-JCSC3624) (data not shown), were obtained from K. Hiramatsu and T. Ito at Juntendo University in Tokyo, Japan (21).

Microtiter plate method

Microtiter plate assay was performed as described by Stepanovic et al. (22), with some modifications. Briefly, 180 μL of tripticase soya broth (Becton Dickinson S.A., 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, the broth was removed and the wells were washed three times with sterile saline to remove nonadherent cells. Plates were air dried in inverted position and bacteria attached were fixed with methanol

for 20 min. Following fixation, microtiter plates were emptied by simple flicking and left to air dry overnight in an inverted position at room temperature. 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 microtiter plate reader Expert Plus – Asys Hitech GmbH (Eugendorf, Austria).

Optical density results were scored and interpreted as described by Stepanovic et al. (22), categorized into strong, moderate, weak producing, and non-producing isolates.

Statistical analysis

Statistical analysis was performed by chi-square test used for comparison of proportion, with significance level of 0.05.

RESULTS

A total of 104 (74 invasive and 30 colonizing) consecutive isolates were analysed. Only a single isolate from each enrolled patient was studied. Seventy invasive isolates were obtained from patients who had both peripheral IVD and blood cultures positive for same staphylococci species. Bacteremia source was considered primary IVD on subjects who had both cultures positive.

Species level identification results showed 27% *S. aureus*, 27% *S. epidermidis*, 6.75% *S. hominis*, and 39.25% other CoNS in invasive group. Only one isolate was identified as *S. aureus* in colonizing group. Antimicrobial resistance profile is demonstrated in Table 1.

The most prescribed antimicrobial for true staphylococci bacteremia was vancomycin (42/74; 56.7%), with therapy period varying between 6 and 19 days. Two patients received vancomycin in combination with linezolid, which had death as clinical outcome. Few patients whose staphylococci isolate belong to colonizing group also received treatment, mainly vancomycin (8/30; 26.7%). The majority remained without any antimicrobial therapy (20/30; 66.7%), at which 40% were biofilm non-producing staphylococci.

The SCCmec typing for 76 mecA-positive isolates was performed. Some isolates were non-typeable with primers utilized in this study (10.5%), but were also capable of biofilm formation (62.5%). Twenty colonizing isolates (74%) harbored SCCmec types IV or V, whereas only 6 (22.2%) harbored SCCmec types I, II, or III. On invasive group, 20% harbored types IV or V ($p < 0.05$). No correlation was observed between biofilm intensity and SCCmec type in both groups (Table 2). Excluding all five MRSA from invasive group, statistical recalculation resulted in same difference (20% invasive strains harboring types IV or V; $p < 0.05$), as they harbored SCCmec types I or III. All positive cultures with staphylococci harboring SCCmec types IV and V isolates positive

Table 1. Overall distribution of antimicrobial resistance phenotype among staphylococci according to each studied group

Antimicrobial tested[2]	Invasive group[1]				Colonizing group[1]		
	SAU n = 20	SEP n = 20	SHO n = 5	oCoNS n = 29	SAU n = 1	SEP n = 18	oCoNS n = 11
	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)
OXA[3]	25	80	80	82.7	100	88.9	91
CIP	25	55	80	79.3	100	77.8	91
CLI	20	50	80	65.5	100	77.8	91
SUT	20	70	80	62	100	77.8	91
DOX	10	10	20	6.9	100	5.5	91
ERY	30	70	80	82.7	100	88.9	91
GEN	25	45	20	75.8	100	66.7	63.6
VAN	0	0	0	0	0	0	0

Resistance profile: R, resistant; given by percentage.
[1]SAU, *Staphylococcus aureus*; SEP, *S. epidermidis*; SHO, *S. hominis*; oCoNS, other coagulase-negative *Staphylococcus*.
[2]OXA, oxacillin; CIP, ciprofloxacin; CLI, clindamycin; SUT, sulfametoxazole-trimethoprim; DOX, doxycycline; ERY, erythromycin; GEN, gentamicin; VAN, vancomycin.
[3]Cefoxitin was used to perform methicillin (oxacillin) resistance.

Table 2. SCCmec types distribution among invasive and colonizing biofilm-producing or non-producing isolates (n = 68)

	SCCmec typing[1,2]							
	Type I n = 17	Type II n = 4	Type III n = 20	Type IV a n = 16	Type IV b n = 2	Type IV c n = 5	Type IV d n = 3	Type V n = 4
Invasive								
Weak	8	–	5	1	1	–	–	–
Moderate	2	2	8	3	1	–	1	1
Strong	3	2	2	1	–	–	–	–
Non-producing	1	–	2	–	–	–	–	1
Total[3]	14 (82)	4 (100)	17 (85)	5 (31)	2 (100)	–	1 (33)	2 (50)
Colonizing								
Weak	1	–	1	4	–	2	1	–
Moderate	–	–	–	2	–	–	–	–
Strong	1	–	1	2	–	2	–	1
Non-producing	1	–	1	3	–	1	1	1
Total[3]	3 (18)	–	3 (15)	11 (69)	–	5 (100)	2 (67)	2 (50)

[1]Only mecA-positive isolates. Non-typeable staphylococci were not demonstrated in this table (n = 8).
[2]One colonizing and two invasive isolates harbored SCCmec I and III simultaneously. Total number of SCCmec types (n = 71) are different from total number of isolates (n = 68).
[3]Total of isolates given in percentage (%) between parentheses.

cultures (30/104) were obtained after at least 10 days from patient hospitalization, mostly over 31 days. Fifteen patients (53.7%) have been previously hospitalizated in the same hospital.

Biofilm formation was observed in 64% of colonizing and 89% of invasive staphylococci, which demonstrated more moderate biofilm formation ($p < 0.05$) (Fig. 1). Significant difference was also observed among staphylococcal species ($p < 0.05$) (Table 3).

DISCUSSION

In this study, the majority of biofilm-producer isolates was multiresistant. Brazilian resistance rates often diverge from worldwide rates, tending to demonstrate high prevalence of multiresistant staphylococci (16, 17), may be due to frequently indiscriminate antimicrobial use in our community set. The SCCmec types IV and V isolates also demonstrated resistance to various antimicrobials other than methicillin in our study, and were found mainly in colonizing CoNS, including *S. epidermidis*. It may raise the hypothesis that the correlation between these cassettes, lower resistance profile, and community origin may not be generalized for all species. Furthermore, the fact that, positive cultures were obtained after at least 10 days from patient's hospital admission and the majority have had previous hospitalization, our results may strengthen this hypothesis. These observations may indicate that SCCmec elements spread and evolution is still ongoing and the relative independence of such elements from bacterium genome also allows them to reside in different species. It can be expected that novel elements may be transferred from CoNS to *S. aureus*. For that reason, antimicrobial resistance evolution in invasive and colonizing biofilm-forming staphylococci is highly relevant (23).

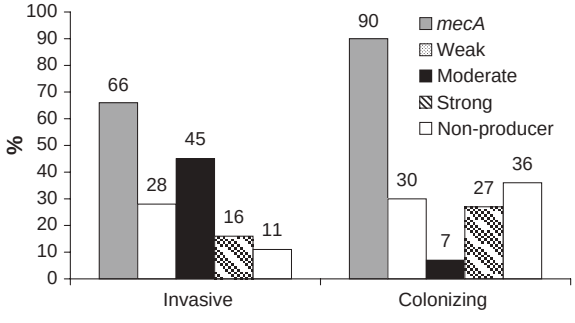

Fig. 1. Biofilm production and *mec* A presence between invasive and colonizing isolates (n = 104).

Colonizing staphylococcal isolates were less frequently biofilm-producers than invasive ones, even considering that CoNS are primarily inhabitants of skin surface, environment where

Table 3. Biofilm production in staphylococci according to group categories and species (n = 104)

	Production of biofilm		
	Invasive b/n[1]	Colonizing b/n[1]	Total b/n (%)[1]
Staphylococcus aureus			
MRSA	5/5	1/1	21/21 (100)
MSSA	15/15	0/0	
S. epidermidis			
MRSE	14/16	13/16	31/38 (81.5)
MSSE	4/4	0/2	
S. hominis			
MRSHo	3/4	0/0	4/5 (60)
MSSHo	0/1	0/0	
Other CoNS			
MRCoNS	21/24	4/10	30/40 (75)
MSCoNS	4/5	1/1	

[1]b total of biofilm-producing isolates, n total of isolates, (%) percentage of biofilm-producing isolates.

these microorganisms are likely to encounter osmotic stress (24) and manifest defense mechanisms, like biofilm (25). Otherwise, all invasive strains were obtained from two consecutive paired blood cultures with a 3-day-interval, and presented high rates of biofilm production. Contrarily to other studies (9, 26, 27), 100% *S. aureus* were biofilm-producers whereas 75.5% CoNS and 81.5% *S. epidermidis*, which originally were described as first slime-producer species (28). Persistent staphylococci bacteremia, commonly defined as bacteremia sustained for 3 days or longer (29) may have biofilm-forming staphylococci as an important risk factor for persistence, mainly when associated with foreign bodies. Nevertheless, previous antimicrobial treatment and biofilm-forming capacity seemed not to be related in this study.

Staphylococcal biofilm formation is complex and heterogeneous, likewise its regulation (3), and distinct behaviors related to invasiveness or colonization potentials are not extensively studied. Biofilm involves diverse proteins and adherence mechanisms, whose molecular expression needs to be clarified. Usual antimicrobials or anti-biofilm molecules may be used as environment stressful agents in each biofilm formation step, to improve an understanding of this bacterial highly organized community.

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7.2 CAPÍTULO 2

Enhancement of antistaphylococcal activities of six antimicrobials against *sasG*-negative methicillin-suscpetible *Staphylococcus aureus*: as in vitro biofilm model

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

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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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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.

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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).

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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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7.3 CAPÍTULO 3

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

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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.

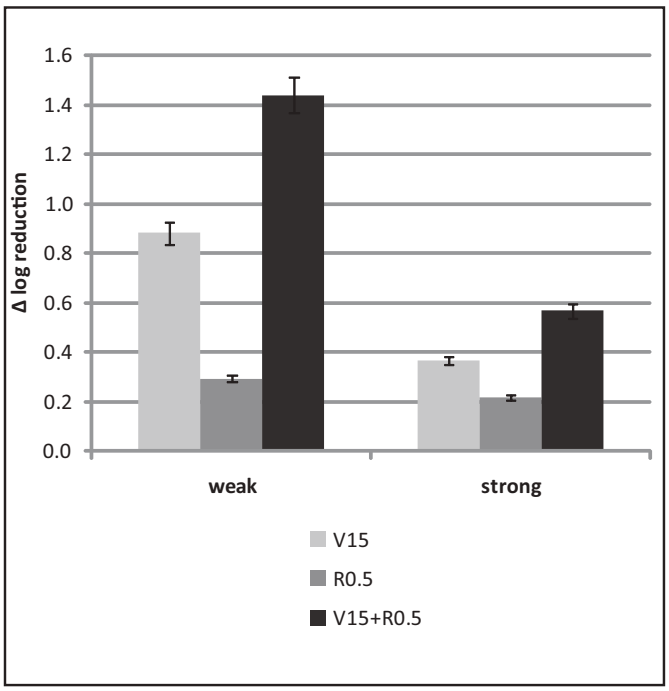

FIGURE 1 - Effect of vancomycin (V15), rifampicin (R0.5), and both drugs in combination (V15+R0.5) against weak and strong MRSA biofilm producers after 24-h exposure.

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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7.4 CAPÍTULO 4

Inhibition of biofilm maturation by linezolid in methicillin-resistant *Staphylococcus epidermidis* clinical isolates: comparison with other drugs

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Inhibition of biofilm maturation by linezolid in meticillin-resistant *Staphylococcus epidermidis* clinical isolates: comparison with other drugs

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Biofilm resistance mechanisms are multifactorial and vary from one organism to another. The purpose of this study was to investigate the efficacy of linezolid against indwelling device-related meticillin-resistant *Staphylococcus epidermidis* (MRSE) biofilm, and compare this with other antimicrobials. MICs, minimum biofilm inhibitory concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs) were determined by the microtitre plate method. Fourteen and thirteen isolates from patients with indwelling device-related bacteraemia (IDB) and indwelling device colonization not associated with bacteraemia, respectively, were assessed. High MBIC was associated with a high intensity of biofilm formation (gentamicin $r=0.796$; linezolid $r=0.477$; rifampicin $r=0.634$; tigecycline $r=0.410$; and vancomycin $r=0.771$), but this correlation was not observed with MBEC. Linezolid demonstrated better *in vitro* antimicrobial activity than other antimicrobials (MBIC – gentamicin $P<0.001$, rifampicin $P=0.019$, vancomycin $P=0.008$; MBEC – gentamicin $P<0.001$, rifampicin $P=0.002$, vancomycin $P<0.001$). Biofilm growth inhibition was strongly associated with biofilm formation intensity; however, biofilm eradication was not cell number dependent. MRSE biofilm eradication would represent a huge advance for IDB, although high concentrations of gentamicin, linezolid, rifampicin, tigecycline and vancomycin were required for that. In general, linezolid reached better *in vitro* concentrations and was demonstrated to be highly active against MRSE biofilms by inhibiting their growth during biofilm formation.

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INTRODUCTION

Staphylococcus epidermidis biofilm creates many barriers against successful antimicrobial therapy, adversely affecting the treatment of indwelling device-related infections by adhering to foreign surfaces and forming a matrix-like coating, preventing immunological factors and antibiotic penetration (Costerton *et al.*, 1999; Mah & O'Toole, 2001; Donlan & Costerton, 2002; Arciola *et al.* 2012). Hence, these infections usually require device removal as well as systemic antimicrobial therapy. However, access vein loss, device replacement and the high cost of this procedure indicate saving the infected device when the clinical situation allows it (Mermel *et al.*, 2001; Arciola *et al.* 2012).

Abbreviations: IDB, indwelling device-related bacteraemia; IDC, indwelling device colonization not associated with bacteraemia; MBEC, minimum biofilm eradication concentration; MBIC, minimum biofilm inhibitory concentration; MRSE, meticillin-resistant *Staphylococcus epidermidis*; ODc, optical density cut-off value.

Bacterial biofilm is highly refractory to antimicrobial treatment, which has serious consequences for the therapy of infections that involve biofilm (Suci *et al.*, 1998). Molecular mechanisms of antimicrobial resistance in biofilm are not the same as for planktonic bacteria, since biofilm formation is accompanied by global genetic regulatory changes. The biofilm lifestyle affords bacteria a 10- to 1000-fold increase in antimicrobial resistance compared to their planktonic counterparts, and many of these genetic changes render the constituent bacteria resistant to antimicrobials (Mah & O'Toole, 2001; Stewart & Costerton, 2001; Patel, 2005; Antunes *et al.*, 2011). The resistance of biofilm to antimicrobials may be associated with limited antimicrobial diffusion through the biofilm matrix (Suci *et al.*, 1994), physiological changes (Dagostino *et al.*, 1991) and a reduced growth rate of bacteria in biofilms (Duguid *et al.*, 1992).

Currently, despite all the biofilm virulence and resistance mechanisms, there is a strong and constant need to find an

antimicrobial that effectively kills biofilm-forming micro-organisms and those already encased in biofilms. The measurement of minimum biofilm eradication concentration (MBEC), and more recently, minimum biofilm inhibitory concentration (MBIC), has been suggested as a laboratory assay to evaluate antimicrobial activity against mature biofilm (Anwar *et al.*, 1990; Sandoe *et al.*, 2002). In the present study, we used an *in vitro* polystyrene microtitre plate biofilm model to determine the MIC, MBIC and MBEC for linezolid against indwelling device-related meticillin-resistant *S. epidermidis* (MRSE) biofilm, and compared these with the results with other antimicrobials.

METHODS

Study design and bacterial strains. *S. epidermidis* strains were recovered from patients attending the Complexo Hospitalar Santa Casa de Misericórdia de Porto Alegre (CHSCMPA), Porto Alegre, Brazil. These strains were recovered from patients with indwelling device-related bacteraemia (IDB) and from patients with indwelling device colonization not associated with bacteraemia (IDC), between August 2010 and January 2011. For IDB, routine blood cultures were performed and the strains were recovered after isolation of the micro-organism in 5 % sheep blood agar. For IDC, routine cultures were performed as described by Maki *et al.* (1977), where colony counts above 15 were considered a positive result.

All isolates were stored at −20 °C and all microbiological analyses were performed at the Gram-positive Cocci Laboratory, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil. All isolates were previously confirmed as *S. epidermidis* using screening and confirmatory methods (Antunes *et al.*, 2008; Bannerman, 2011), and screened for meticillin resistance using 30 µg cefoxitin discs (CLSI, 2011); this was confirmed further by the presence of *mec*A (Zhang *et al.*, 2005).

Group definitions. Bacteraemia was defined as ⩾2 consecutive 3 day-interval paired positive blood cultures with MRSE. IDB was defined as a bacteraemia where the primary source of the infection was the indwelling device, in patients with temperature >38 °C, chills and septic appearance. IDC was defined as a positive culture and a negative peripheral blood culture for the same micro-organism.

Antimicrobials. Gentamicin, linezolid, rifampicin and vancomycin analytical powders were provided by Sigma-Aldrich. Tigecycline powder was a gift from Wyeth Pharmaceuticals.

Biofilm determination and quantification. Biofilm formation ability was determined by microtitre plate assay, and optical density results were scored and interpreted as described elsewhere (Stepanović *et al.*, 2007). Briefly, 180 µl 1 % glucose-tripticase soya broth (TSB) (Becton Dickinson) was added to a sterile 96-well polystyrene flat-bottom microtitre plate (TPP Techno Plastic Products), followed by 20 µl 1×10^8 c.f.u. ml^{-1} bacterial suspension (a total of 1×10^7 c.f.u. ml^{-1}). The plates were incubated for 24 h at 35±2 °C under static conditions. After incubation and broth removal, the plate was washed three times with sterile saline, and attached bacteria were fixed with methanol and left to air dry overnight in an inverted position. Finally, adherent bacteria were stained with 0.5 % crystal violet.

To quantify biofilm, optical density was measured at 492 nm using the Expert Plus microtitre plate reader (Asys Hitech). The optical density cut-off value (ODc; negative control optical density at 492 nm) was defined as threefold the SD above the negative control (in practical terms, a reading around 0.090 at 492 nm) and the intensity of biofilm formation was categorized as strong ($2ODc\leqslant OD_{492}\leqslant 4ODc$) or weak ($ODc<OD_{492}<2ODc$).

MIC determination. Conventional MICs of gentamicin, linezolid, rifampicin, tigecycline and vancomycin were determined in all isolates in duplicate, by twofold serial broth microdilution (CLSI, 2012). *Staphylococcus aureus* ATCC 29213 was tested as a quality control.

MBIC and MBEC determinations. MBIC and MBEC experiments were performed using a modified version of the Calgary biofilm device method (Ceri *et al.*, 1999; Cafiso *et al.*, 2010). In brief, 20 µl 10^8 c.f.u. ml^{-1} bacterial suspensions were added to 180 µl 1 % TSB, placed into a sterile 96-well polystyrene flat-bottom microtitre plate (TPP Techno Plastic Products) and incubated overnight at 35 °C without shaking, to allow bacterial attachment. Non-adherent cells were removed by gentle washing three times with sterile saline solution (150 µl 0.9 % NaCl). The plates were left to air dry for 15 min. Serial twofold dilutions of each antimicrobial agent in cation-adjusted Mueller–Hinton broth (CAMHB) were added to the microplates followed by incubation at 35 °C for 24 h. MBIC was defined as the minimal antimicrobial concentration at which there was no observable bacterial growth in wells containing adherent microcolonies, i.e. the minimal concentration that inhibited the release of planktonic bacteria from biofilm.

After MBIC measurement, the broth was removed and wells were washed three times with sterile saline solution (150 µl 0.9 % NaCl) and antimicrobial-free CAMHB added, 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.

Statistical analysis. Mann–Whitney U and Spearman rank correlation tests were used for continuous non-normally distributed data and multiple comparisons were performed using the Kruskal–Wallis test followed by Dunn's post hoc test for simultaneous pairwise inference. Differences were considered statistically significant at $P\leqslant0.05$. All statistical tests were performed using SPSS software version 16.0 (SPSS).

RESULTS AND DISCUSSION

S. epidermidis infections are considered extremely recalcitrant to therapy mainly due to treatment failure associated with its ability to form biofilm in medical devices, from where these multilayered bacteria aggregates are very hard to remove (Mack *et al.*, 2006). When it comes to biofilm, several parameters must be evaluated regarding antimicrobial resistance. With the advent of multidrug-resistant micro-organisms and recurring treatment failures caused by biofilm-forming bacteria, new treatment approaches must be assessed to improve outcomes. This study was mainly designed to evaluate linezolid activity against MRSE biofilm.

A total of 38 consecutive *S. epidermidis* isolates were recovered; 20 were obtained from patients with IDB and 18 from patients with IDC. Among IDB isolates, four were meticillin susceptible and two did not form biofilm. Among IDC isolates, two were meticillin susceptible and three did not form biofilm. These 11 isolates were excluded from the study and the remaining 14 IDB-MRSE and 13

IDC-MRSE isolates were assessed. An explanation for these non-biofilm-forming isolates may be phase variation, that is one of the strategies employed by pathogenic bacteria to switch on the expression of proteins according to the environment. So, even if these non-biofilm-forming isolates harboured the genetic ability to produce biofilm, it does not necessarily mean that biofilm will be produced (Conlon *et al.*, 2004; Tormo *et al.*, 2007).

There was no difference between the groups (IDB and IDC) regarding MIC, MBIC and MBEC. All MRSE were susceptible to linezolid, tigecycline and vancomycin, 18 were susceptible to rifampicin (66.7 %) and 12 to gentamicin (44.4 %) by MIC determination (Table 1). These susceptible isolates were then submitted to antimicrobial exposure after *in vitro* adherence on polystyrene microtitre plates and the results were extremely high when compared to the MIC. MBEC varied from one to seven dilutions higher than MBIC. The MBIC and MBEC for other antimicrobials were statistically higher when compared with linezolid, except for tigecycline in which no difference was observed (Table 1). Linezolid resistance has remained relatively uncommon among staphylococci. However, recent reports of linezolid resistance among coagulase-negative *Staphylococcus* (CNS) at medical centres raises concerns (Potoski *et al.*, 2006; Dandache *et al.*, 2009). Because of the high prevalence of CNS biofilm in our setting (Antunes *et al.*, 2010, 2011; Reiter *et al.*, 2011) and around the world (Dandache *et al.*, 2009; Fredheim *et al.*, 2009; Jain & Agarwal, 2009), and the increasing prevalence of higher antimicrobial resistance rates among these biofilm-forming isolates (Cha *et al.*, 2011), future potential treatments for staphylococcal infections mediated by the formation of biofilms are compromised.

MBEC/MIC and MBIC/MIC ratios were calculated to verify how much higher the antimicrobial concentrations were when tested against adherent cells in comparison with planktonic cells. These ratios describe the importance of antimicrobial concentration detection in biofilm, since the results demonstrated an estimation of significant differences between planktonic and sessile cells in terms of antimicrobial performance. Rifampicin presented the highest ratios, followed by gentamicin, as shown in Fig. 1. Both graphs correlate the cumulative number of isolates with each ratio value, and it was observed that linezolid, tigecycline and vancomycin demonstrated statistically lower ratios than rifampicin and gentamicin, evidenced by each antimicrobial curve tendency (Fig. 1). A lower slope of the curve indicated a worse performance of the correspondent antimicrobial against biofilm, i.e. it is necessary to use higher concentrations ($P<0.001$). The impact of these ratios is evident when treatment failure occurs in cases where physicians have prescribed appropriate antimicrobial doses.

Linezolid presented better concentrations against adherent cells than other antimicrobials tested, even when compared to vancomycin. These results raise many questions and worries about the questionable activity of vancomycin against biofilm, which was already demonstrated to be better than daptomycin, tigecycline, ceftriaxone and azithromycin (Presterl *et al.*, 2009) and worse than rifampicin and ciprofloxacin (Qu *et al.*, 2009, 2010). We found that vancomycin activity was similar to linezolid and tigecycline, and higher than gentamicin and rifampicin activities against MRSE biofilm. However, linezolid seemed to be a more indicated treatment option against biofilm, as seen in other studies (Rodríguez-Martínez *et al.*, 2007; Bayston *et al.*, 2012).

It is important to know the biofilm-producing ability and micro-organism at the species level, since there may exist critical differences between these features. Antunes *et al.* (2010) demonstrated that MIC results for staphylococci did not change when the isolate was not capable of biofilm

Table 1. MIC, MBIC and MBEC results of planktonic and adherent MRSE isolates

Antimicrobial agent	Planktonic bacteria			Adherent bacteria*					
	MIC range	MIC_{50}	MIC_{90}	MBIC range†	$MBIC_{50}$	$MBIC_{90}$	MBEC range‡	$MBEC_{50}$	$MBEC_{90}$
Gentamicin	<0.125–>16	<0.125	>16	8–256	32	128	64–256	64	256
Linezolid	0.125–1	0.5	1	2–128	4	64	4–256	32	128
Rifampicin	<0.03–32	0.06	32	2–128	32	64	16–256	64	256
Tigecycline	0.06–2	1	2	2–256	8	64	4–256	16	128
Vancomycin	0.5–4	1	2	4–256	16	128	32–256	128	256

*MBIC and MBEC determination was performed only for isolates under each antimicrobial breakpoint (susceptible by MIC): gentamicin $n=12$; linezolid $n=27$; rifampicin $n=18$; tigecycline $n=27$; vancomycin $n=27$.

†Statistically significant differences were found between linezolid MBIC values and gentamicin ($P<0.001$), rifampicin ($P=0.019$) and vancomycin ($P=0.008$) MBIC values.

‡Statistically significant differences were found between linezolid MBEC values and gentamicin ($P<0.001$), rifampicin ($P=0.002$) and vancomycin ($P<0.001$) MBEC values. There was no significant difference between linezolid and tigecycline MBIC ($P=0.148$) and/or MBEC ($P=0.278$) values.

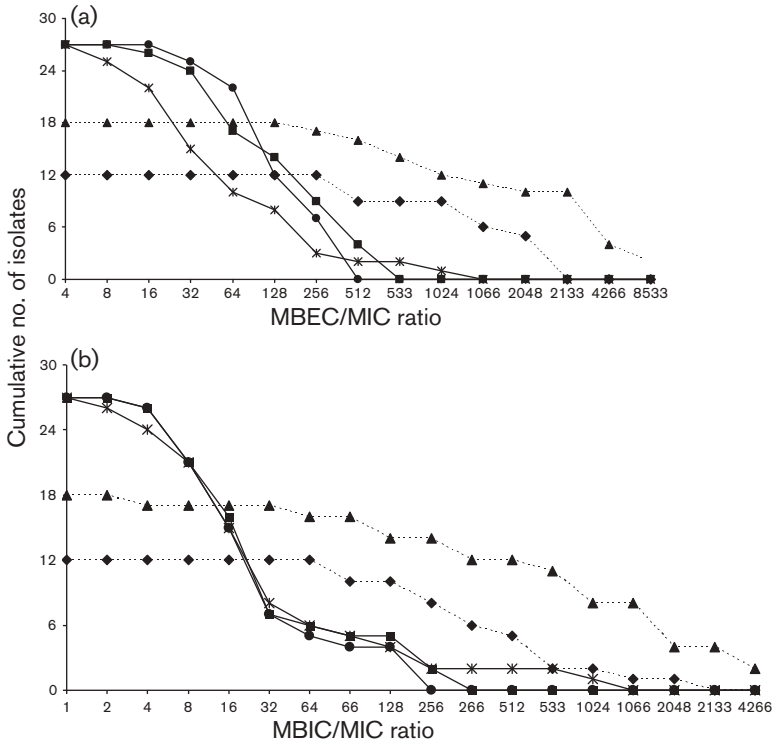

Fig. 1. Comparison between each antimicrobials tested and their respective MBEC/MIC (a) and MBIC/MIC (b) ratios. The cumulative number of isolates shows the antimicrobial efficiency pattern, a greater efficiency is demonstrated by the earlier dropping of the curve for linezolid, tigecycline and vancomycin. Statistically significant differences were found between the following pairs: linezolid compared with gentamicin, linezolid compared with rifampicin; tigecycline/gentamicin, tigecycline/rifampicin; vancomycin/gentamicin and vancomycin/rifampicin ($P<0.001$). ◆, Gentamicin; ■, linezolid; ▲, rifampicin; X, tigecycline; ●, vancomycin.

production. Likewise, Raad *et al.* (2007) showed that linezolid and vancomycin were less effective against meticillin-resistant *S. aureus* biofilm than other antimicrobials, as also demonstrated by Rose & Poppens (2009), but not as demonstrated by our study with MRSE.

Spearman's coefficients from correlation between the MBICs and the intensity of biofilm formation (the OD_{492} reading) demonstrated regular and strong correlation for all antimicrobials (Fig. 2). Weak biofilm intensity was associated with lower MBICs for all antimicrobials.

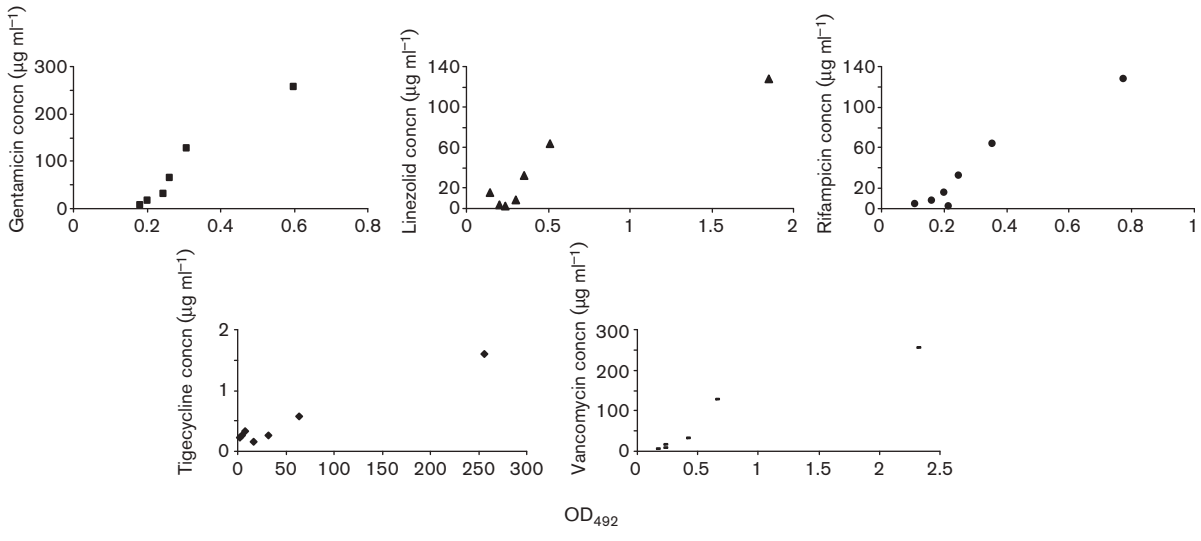

Fig. 2. Correlation between each antimicrobial MBIC (μg ml^{-1}) and OD_{492}. Spearman's coefficients were calculated: gentamicin, $r=0.796$ $P=0.002$; linezolid, $r=0.477$ $P=0.012$; rifampicin, $r=0.634$ $P=0.005$; tigecycline, $r=0.410$ $P=0.034$; and vancomycin, $r=0.771$ $P<0.001$. Each point refers to the mean OD_{492} at each MBIC.

However, even linezolid demonstrated lower MBICs compared with other antimicrobials, the correlation between these MBICs and intensity was poor. Rodríguez-Martínez *et al.* (2007) demonstrated that linezolid penetration in *S. epidermidis* biofilms was significantly greater than vancomycin, as well as other studies that have demonstrated a reduced vancomycin penetration through *S. aureus* and *S. epidermidis* biofilms (Jefferson *et al.*, 2005; Singh *et al.*, 2010).

However, when the MBECs and the intensity of biofilm formation were compared, there was no statistically significant correlation among the antimicrobials (gentamicin $r=0.298$, $P=0.06$; linezolid $r=-0.009$, $P=0.966$; rifampicin $r=0.237$, $P=0.344$; tigecycline $r=0.345$, $P=0.078$; and vancomycin $r=0.082$, $P=0.684$). Complete biofilm eradication was not associated with biofilm intensity and this difference cannot be explained by the present study. Maybe biofilm growth inhibition is more likely to be related to cell number because the majority of these cells could be at their basal metabolism status, surviving in the environment thanks to the biofilm community. Then, after therapy discontinuation, these cells could repopulate the biofilm, independently of the antimicrobial concentration that has been applied to it.

There are a lot of contradictory facts about the behaviour of staphylococcal biofilms in response to antimicrobials, and our study helps to show how difficult it is to treat bacteria living in biofilms considering our results. Due to inherent resistance to antimicrobial agents, it is extremely important to reach an effective antimicrobial concentration at the treatment site. Although no antimicrobial provides complete biofilm eradication, linezolid seemed to be highly active against MRSE biofilm by inhibiting its growth at reachable concentrations.

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7.5 CAPÍTULO 5

Influence of vancomycin and linezolid in the expression of biofilm-associated genes in *Staphylococcus epidermidis*

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Influence of vancomycin and linezolid in the expression of biofilm-associated genes in *Staphylococcus epidermidis*

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ABSTRACT

Biofilms are complex polymeric structures that allow the bacteria to survive in hostile environments. They are more resistant to antibiotics and therefore are more difficult to eradicate. The aim of the study was to investigate the influence of vancomycin and linezolid on the maintenance of staphylococcal biofilms and their effect on the expression of biofilm-associated genes in *S. epidermidis*. Preformed biofilms of *S. epidermidis* RP62A were challenged by linezolid and vancomycin with different concentrations and also with the clinical concentration goal (15 μg/ml) over the time. The expression of *icaA, atlE, aap, rnaIII, luxS, sarA, rsbU* and *icaR* genes after 2 hours of exposure to these antibiotics was determined by Qpcr Vancomycin did not affect significantly the biofilm under the tested conditions, but linezolid affected the biofilm structure with concentrations equal or above 2 μg/ml ($p<0.05$). Also, the exposure time to this antibiotic was determinant for biofilm eradication. The level of transcription of *icaA, aap* and *atlE* increased by 5.18-fold, 2.58-fold and 3.06-fold, respectively, in the biofilms exposed to linezolid but no changes were found for vancomycin. The other genes were not affected by these antibiotics. Our study demonstrated that linezolid was effective to eradicate biofilms formed by *S. epidermidis* RP62A. At the conditions tested, linezolid up-regulated biofilm-associated genes probably due to the stress caused by antibiotic low-dose stimulation. In our study, linezolid showed better performance than vancomycin against staphylococcal biofilms.

INTRODUCTION

Staphylococcus epidermidis is widely known for causing foreign device-related infections, which are attributed primarily to its ability to live as biofilms on a polymer surface (1). Biofilms constitute complex polymeric structures that allow bacteria to survive in hostile environments (2). In *Staphylococcus*, the biofilm formation relies on a successful initial adherence and further accumulation of cell layers (3). During the first step, the bacteria interact with the solid surface through nonspecific electrostatic forces and hydrophobic effect. Subsequently, the biofilm progressively develops on the colonized surface, forming the basis building block or structural unit of the biofilm, the microcolony (4,5). Its maintenance depends on the formation of an exopolysaccharide matrix (EPS), which is mainly composed of a homopolymer denominated PIA (polysaccharide intercellular adhesin) (6). Its production is responsible for the biofilm architecture and prevents bacterial cells of being exposed to antibiotics due to chemical and physical mechanisms, leading to a concentration gradient of substances along the biofilm matrix. Hence, the cells on the external layers are the most affected by antibiotics and those called persister cells, more closely attached to the surface, survive (6,7). The fundamental requirement for the permanence of cells in biofilm is the presence of these latter cells, which are recalcitrant to antibiotic treatment without acquiring heritable resistance (7).

Different environmental conditions influence the formation of staphylococcal biofilms, therefore a complex regulatory system underpins this process (7,8). In regards to biofilm regulation, *S. epidermidis* relies on a quorum-sensing response based on the production and recognition of peptide-based pheromones, which allows the pathogen to adapt to the changing environmental conditions such as antibiotic exposure. Besides regulating biofilm formation, the system regulates virulence factors and, therefore, is crucial for the development of the disease (9-11).

Studies have shown that sub-inhibitory concentrations of antibiotics (e.g. gentamycin, rifampicin and tigecycline) can influence the expression of genes in *Staphylococcus aureus* and *S. epidermidis*, which may alter the outcome and the treatment of infections related to biofilm formation (12-15). The investigation of the influence of the antibiotics on the biofilm-associated genes expression and phenotypic patterns is one step forward for the understanding of the bacterial response to the antibiotic stress. Consequently, the best practices to circumvent the action of these pathogens could also be determined. Our study evaluated the influence of vancomycin and linezolid, antibiotic choices for treatment of staphylococcal infections, on the expression of genes associated with biofilm formation in *S. epidermidis*.

MATERIAL AND METHODS

Strain and antibiotics

Staphylococcus epidermidis RP62A (ATCC 35984) was the biofilm-producing strain used in the study. For biofilm challenge, the experiments were conducted with vancomycin and linezolid (Sigma-Aldrich, St. Louis, MO, USA) with their clinical concentration goal (15 μg/ml) (16,17).

Determination of biofilm density after antibiotic challenge

The biofilm promotion was performed as described previously by Christensen *et al.* (1985) and Stepanovic *et al.* (2007), with some modifications (18,19). Briefly, 20 μl of $1x10^8$ CFU/ml bacterial suspension was inoculated in each well of a sterile 96-well polystyrene flat-bottom microtiter plate (TPP Techno Plastic Products, Trasadingen, Switzerland) containing 180 μl of 1% glucose-trypticase soy broth (Becton Dickinson S.A., Franklin Lakes NJ, USA). The bacterial culture was incubated for 24 h at 35°C under static conditions to allow the biofilm formation. Subsequently, the antibiotic challenge was performed. For this purpose, the broth was removed and replaced by cation-adjusted Mueller Hinton broth (CAMHB) with or without antibiotic. The antibiotic challenge of the biofilms was conducted with vancomycin or linezolid (Sigma-Aldrich, St. Louis, MO, USA), using their clinical concentration goal (15 μg/ml) (16,17). A negative control using drug-free CAMHB was also perfomed. The plates were incubated for 0 h (no incubation), 2 h, 6 h, 10 h and 24 h at 35°C. At each different time, the colorimetric assay was conducted as described in Stepanovic *et al.* (2007): the CAMHB was removed, the plate was washed three times with sterile saline and the biofilm was fixed with methanol and left to air dry overnight in inverted position. Finally, the biofilm was stained with crystal violet 0.5%. For optical density reading, ethanol 95% was added to the stained biofilm and the absorbance was measured at 492 nm using microtiter plate reader 120 Expert Plus - Asys Hitech GmbH (Eugendorf, Austria). Three independent cultures were analyzed in each antibiotic challenge time and the OD_{492} was determined using the negative control (without bacteria) (19).

After that, a challenge using a concentration gradient (0.5, 2, 8 and 15 μg/ml of each antibiotic) was performed. The assay was conducted as described above, in triplicate, but with incubation only for 24 h to evaluate in which concentration the biofilm formation could be more affected by sub-inhibitory concentrations of vancomycin or linezolid. All analyses were performed using Prism software (GraphPad Software, Inc.).

Biofilm growth and antibiotic challenge for gene expression analysis

For the gene expression analysis, in order to maximize the RNA yield, the antibiotic challenge of the biofilms was performed using polystyrene plates with 90 mm x 15 mm, which allowed a greater extension for biofilm formation. Twenty ml of 1% glucose-tripticase soya broth (Becton Dickinson S.A., Franklin Lakes NJ, USA) was added to each plate followed by 120 µl of $1x10^8$ CFU/ml bacterial suspension. The plates were incubated for 24 h at 35°C. Then, the broth was removed and replaced with 20 ml of CAMHB without antibiotic (control free-drug biofilm) or 20 ml of CAMHB with vancomycin or linezolid at 15 µg/ml. The plates were incubated for 2 h at 35°C. After incubation, the broth was removed for RNA isolation. Five independent cultures were utilized for the gene expression analysis.

RNA isolation, quantification and purification

Total RNA of each sample was extracted using Trizol® reagent (Invitrogen, Paisley, United Kingdom) following the manufacturer's manual. After the biofilms were rinsed, each biofilm was immediately ressuspended in 1 ml of cold sterile ultra-pure water by flushing the plates with water. Then, using a sterile spatula, the biofilm was removed from the plates. Biofilm cells were centrifuged for 5 min at 3,345 x g at 4°C and the pellets were weighted (average yield of 50.1 mg; 39.2 to 61.7 mg) and then ressuspended directly in 1 ml of Trizol. The RNA samples were ressuspended in 20 µl of RNAse-free water.

The RNA was quantified using BioSpec-nano Spectrophotometer (Shimadzu, Tokyo, Japan) and the purity was evaluated through the absorbance ratio A260/A280 (the average value of the samples was 1.90 (1.74 to 2.09)). The average amount of acid nucleic yield was 105.76 ng/µl (60.24 to 178.64 ng/µl).

To remove DNA contamination, all of the RNA samples were digested with DNase I (Promega, Madison WI, USA). For about 1 µg of total RNA, it was added 2 U of DNAse I and incubated at 37°C for 30 min. Subsequently, one microliter of DNAse Stop solution was added and the reaction was incubated at 65°C for 10 min. The quality of the RNA after the DNAse treatment was not assessed due to the low RNA yield.

cDNA synthesis

Eight microliters of the DNAse-treated RNA solution was utilized for cDNA synthesis, which was perfomed by random hexamers priming with the SuperScript TM First-Strand Synthesis System for RT-PCR (Invitrogen, Paisley, United Kingdom), according to the manufacturer's instructions. For each RNA sample, a mock reaction without reverse transcriptase (RT-) was performed for further evaluation of gDNA contamination.

Primer design

Primers for qPCR were designed using Primer3 web-based software (http://fokker.wi.mit.edu\cgi-bin\primer3\primer3_www.cgi) and are listed in Table S1. Each primer pair was designed considering thermodynamic and sequence criteria as amplicon lenght of 50-210 bp, primer lenght of 19-23 nucleotides, GC content of 35-65%, Tm of 60-68°C and 3'-end stability.

Quantitative real-time PCR

For each of the 12 genes (*aap, atlE, icaA, icaR, luxS, gyrB, DHFR, rnaIII, sarA, rsbU, tpiA* and *16S*), the qPCR amplification was performed with 1 µl of a 1:30 dilution of the DNase-treated cDNA, 10 µl of SYBR Green qPCR Master Mix (GoTaq, Promega, Madison, WI, USA), 1 µl of forward primer (4 µM), 1 µl of reverse primer (4 µM) and RNase- DNase-free water added to a final volume of 20 µl. The qPCR was carried-out by using the 7500 Real-Time PCR System (Applied Biosystems, California, USA), with the following cycle parameters: holding stage of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and then 60°C for 60 s with a final melting curve determination. All qPCR were performed in triplicate for each of cDNA samples. A difference of at least 7 Ct between the cDNA sample and the RT- sample or negative control PCR was considered negligible for the relative quantification analysis. Amplicons were sequenced using the ABI 3130 Genetic Analyzer platform (Applied Biosystems, California, USA) and the obtained sequences were compared with the target genes of the *S. epidermidis* RP62A genome (accession number NC_002976.3) through BLAST.

Reference gene selection

In order to choose the best reference gene for normalizing the data obtained from qPCR, four housekeeping genes (*tpiA, gyrB*, *16S* and *DHFR*) were selected. For each gene, the qPCR amplification was performed with the parameters described above. PCR efficiency was estimated using LinRegPCR software version 2012.3 (20) and the average PCR efficiency of all genes was 1.944. Four software programs, geNorm version 3.5, Normfinder version 0.953, Bestkeeper version 1 and the comparative dCt method were used to evaluate the stability of the candidate reference genes (21-24). The analyzed data was derived from five independent experiments.

Relative expression

Average Ct and the cycle threshold values for each gene were obtained using 7500 software version 2.0.6 (Applied Biosystems, California, USA) and the average efficiencies were calculated using LinRegPCR software version 2012.3 (20).

184 The relative expression of the target genes in the antibiotic condition in relation to the drug-
185 free control was determined using REST 2009 (version 1). The significance of the relative
186 quantification was tested by a pair-wise randomization test from five independent experiments (25).
187
188 **RESULTS**
189
190 **Influence of vancomycin or linezolid on the biofilm density over the time**
191 The ability of vancomycin or linezolid to affect the biofilm density over the time was
192 observed through the exposure of preformed biofilms to each antibiotic at 15 μg/ml. At time 0, no
193 significant differences were observed on the biofilm density among all treatments. In the drug-free
194 control and vancomycin treatment, the density of the biofilms increased over the time. No
195 statistically significant differences were observed in relation to the density presented by the
196 vancomycin-treated and control drug-free biofilms in each time. On the other hand, when biofilms
197 were exposed to linezolid, the density of the biofilm gradually decreased over the time. The
198 difference between the linezolid-treated and control drug-free biofilms was statistically significant
199 in 2, 6, 10 and 24 h. Vancomycin-treated cells remained adherent to the wells over the time,
200 whereas linezolid-treated cells floated between adherent and non-adherent (Fig. 1), visualized
201 through the OD reading below the negative control (data not shown). However, no statistically
202 significant growth differences were verified among the observed times.
203
204 **Influence of vancomycin or linezolid concentration on the biofilm density**
205 The effect of different concentrations (0, 0.5, 2, 8 and 15 μg/ml) of vancomycin or linezolid
206 on the biofilm maintenance after 24 hours of exposure was evaluated. For the vancomycin
207 treatment, concentrations up to 15 μg/ml did not influence the density of the biofilm (Fig. 2). On the
208 other hand, concentrations equal or above 2 μg/ml of linezolid affected the biofilm structure (Fig.
209 2). Similarly to the evaluation of biofilm density over the time, linezolid-treated cells reached non-
210 adherent characteristics above 0.5 μg/ml, visualized when the OD reading was below the negative
211 control (data not shown).
212
213 **qPCR optimization and normalization**
214 Precise quantification in qPCR assays is dependent of high PCR efficiencies, which vary for
215 each primer pair. Therefore, the PCR efficiency for each gene was determined using the
216 LinRegPCR software. The average efficiencies were 1.92 for *icaA*, 1.93 for *aap*, 1.92 for *atlE*, 1.96
217 for *rnaIII*, 1.94 for *sarA*, 1.97 for *luxS*, 1.98 for *icaR*, 1.96 for *rsbU*, 1.95 for *gyrB*, 1.92 for *16S*,
218 1.89 for *tpiA* and 1.95 for *DHFR*. The melting curve analysis displayed a single sharp peak for each

target gene, and the primers specificity was also validated by sequencing. All sequences evaluated matched the correct genes of *S. epidermidis* RP62A strain genome available in GenBank database, demonstrating the specificity of the designed primers and the genes of interest.

The choice of the best reference gene for this experimental study was conducted and the stability evaluation of each gene was analyzed through four different approaches (Fig. 3). The expression level of *16S* was greater than all other housekeeping genes (average Ct of 15.24 compared to 26.14, 26.61 and 24.66 of *tpiA*, *gyrB* and *DHFR*, respectively). The Bestkeeper software could not be employed in this analysis because the standard deviation for all four housekeeping genes was greater than 1, despite high coefficients of correlation with the Bestkeeper (0.962 to 0.988; $p<0.001$). According to Normfinder and dCt method, *16S* was the most stable gene, followed by *gyrB*, *tpiA* and finally *DHFR* (Fig. 3). Despite geNorm demonstrated *tpiA* as second most stable gene, *gyrB* was also suitable as reference gene. All genes were stable to be used as reference and since *gyrB* is frequently selected for this purpose, it was selected for this study.

Influence of vancomycin and linezolid on the expression of effector genes *icaA*, *aap*, *atlE* and *rnaIII* and regulatory genes *luxS*, *rsbU*, *sarA* and *icaR*

The relative expression of specific biofilm-associated genes was determined using qPCR. The expression of each gene was evaluated after exposition of preformed biofilms to a concentration of 15 μg/ml of vancomycin or linezolid for 2 hours. The level of transcription of *icaA*, *aap* and *atlE* increased by 5.18-fold, 2.58-fold and 3.06-fold, respectively, in the linezolid-treated biofilm in relation to the drug-free biofilm ($p<0.05$) (Fig. 4A). Similarly, *icaA* was increased by 1.88-fold in vancomycin-treated cells in comparison to the level of transcription in the drug-free control, although it showed no significantly changes as also *aap* and *atlE* genes (Fig. 4B). No significant alteration on the expression of *rnaIII* gene was detected in the linezolid-treated or in the vancomycin-treated biofilms when compared with the drug-free biofilm. The level of transcription of the regulatory genes *sarA*, *luxS*, *rsbU* and *icaR* was slightly altered when the biofilms were exposed to vancomycin (1.23-fold, 1.38-fold, 1.23-fold and 0.97, respectively), but this variation was not statistically significant. Likewise, in the linezolid-treated biofilms, the changes in the expression level of these genes were also not significant (Fig. 5A, Fig. 5B).

DISCUSSION

Our study investigated the influence of the clinical concentration of two important antibiotics, vancomycin and linezolid, on the maintenance of staphylococcal biofilms, as well their effect on the expression of biofilm-associated genes.

In general, *Staphylococcus* spp. planktonic cells are inhibited by vancomycin and linezolid at concentrations up to 4 μg/ml (26). On the other hand, when bacteria are in biofilm, the inhibitory concentrations increase 10-1000 fold (27). Various studies have already demonstrated how higher the concentrations of diverse antibiotics should be to eradicate the cells in biofilm (28-32).

Our study demonstrated that increasing concentrations of vancomycin up to 15 μg/mL did not influence the biofilm structure of *S. epidermidis* RP62A. In fact, using the clinical concentration goal, vancomycin was not able to eradicate biofilm even within 24 hours of exposure and did not affect significantly the development of the pre-formed biofilm. Vancomycin is a glycopeptide with high molecular weight, and the lack of activity on biofilms could be associated mainly with its limited diffusion through the biofilm matrix due to the sequestration by the biofilm components (33,34). Jefferson *et al.* (2005) demonstrated by confocal microscopy, that vancomycin bind to free-floating bacteria in water within 5 min, but more than 1 hour to cells immersed in the deepest layers of a biofilm. This observation demonstrates that the vancomycin mechanism of action is impaired by its reduced rate of penetration (35).

In contrast to vancomycin, in this study it was demonstrated that linezolid was more effective in inhibiting the biofilm formation, since lower concentrations of this antibiotic (above 2 μg/ml) were able to eradicate the biofilm. When the clinical concentration was used, the biofilm was eradicated after 6 h of exposition. One possible explanation for these observations could be related to its better ability of diffusing in the biofilm matrix than vancomycin, since it has a lower molecular size (33,36).

The regulation system of biofilm formation is complex, and is responsible for the transcription of genes encoding adhesins and the polysaccharide PIA (6,11). The *atlE, icaA* and *aap* genes play an important role in the biofilm formation in staphylococci (11,37,38). Vancomycin did not have influence on the expression of these genes, and it probably occurred due to the inability of this antibiotic to permeate the biofilm, as discussed previously. It is likely that vancomycin did not reach the cells in biofilm, not causing stress, and consequently it did not require an effective response by the cells. On the other hand, linezolid affected the expression of *atlE*, *icaA* and *aap* genes. Although biofilms are interspersed by open channels, the matrix itself hampers the free diffusion of antibiotics throughout the biofilm. Therefore, there is a concentration gradient along the biofilm and at the basal cells, which are intimately attached to the surface, the antibiotic concentrations are low (4,33). As the linezolid penetration rate through the biofilm is lower than in planktonic cells, the bacteria may have been exposed to a gradual increasing dose of antibiotic and may have had the time to mount a defensive response to the compound (39,40). Therefore, since after 2 h the biofilm was not eradicated, the exposition to low doses of linezolid led to an increased expression of the biofilm-associated genes *icaA*, *atlE* and *aap*. Gomes *et al*. (2011) demonstrated

that the expression of genes as *icaA* and *rsbU* increased after exposure to rifampicin and gentamicin, but antibiotic combinations induced a lower expression of these genes. Sub-inhibitory toxic conditions, as antibiotic exposure, has been reported to be inductors of biofilm formation and promote expression of genes by surviving cells (12,14). However, high-dose of antibiotics has an opposite effect on the biofilm, since it leads to the dispersion of the bacterial cells (41-43). The term hormesis is used generally to describe this phenomenon characterized by biphasic dose-response relationships exhibiting low-dose stimulation and high-dose inhibition (40).

In order to establish a biofilm-associated clinical condition, *S. epidermidis* coordinate the expression of different genes, which allows the pathogen to adapt and maintain the infection. Two quorum-sensing systems, the accessory gene regulator (*agr*) and *luxS*, coordinate the process (11,44). The Agr system down-regulates SarA, which is a global regulatory protein that promotes the expression of the *ica* operon. This operon is responsible for encoding the PIA, the main protein involved on biofilm matrix composition. The *rsbU* gene also stimulates the biofilm formation, influenced indirectly by QS systems, and positively regulates the stress sigma factor B, which controls the *ica* operon expression by repressing the transcription of *icaR* via a regulatory cascade. Moreover, the *sarA* locus is transcribed from three independent promoters, of which the first one is sigma B-dependent (6,45). Our study did not found differences on the expression of the regulatory genes *sarA*, *luxS*, *rsbU* and *icaR* after 2 h of exposition to antibiotics. Since *atlE*, *icaA* and *aap* were active in the conditions tested and are positively controlled by *sarA* and *rsbU* (46), one might expect that these latter regulatory genes would have their expression up-regulated. A variation on the expression of *sarA* and *rsbU* genes was not detected maybe due to an hierarchical response to the stress condition, since they would be primarily expressed. As expected, *luxS* and *icaR* did not have an up-regulation on their expressions because these genes appear to be important *ica* operon down-regulators in *S. epidermidis* (45).

Once biofilm reaches its final phase of maturation, the response of autoinducing peptides triggers the onset of the dispersal phase, characterized by the increase of RnaIII expression (6). Our results did not demonstrate variation at *rnaIII* expression on biofilm exposed to linezolid or vancomycin, and this was supported by the up-regulation of biofilm-related genes. However, environmental signals of sub-inhibitory concentrations of antibiotics that inhibit ribosome function, like linezolid, have a general inhibitory effect on the transcription of *rnaIII* (41).

S. epidermidis utilizes the biofilm mode of growth to initiate and establish infections that became recalcitrant to antibiotic therapy. Our study demonstrated that 2 mg/mL of linezolid is sufficient to eradicate the biofilms of *S. epidermidis* RP62A. The exposure to the clinical concentration goal of linezolid (15 mg/mL) for 2 hours stimulated the overexpression of biofilm-associated genes, but this stress response was not effective, since an increasing on the biofilm

density was not observed in the subsequent hours. Therefore, these results reinforce that linezolid is a proper antibiotic candidate for the treatment of *S. epidermidis* infections. Vancomycin did not affect the biofilm density and also did not have effect on gene expression. Probably due to its chemical properties this antibiotic seemed to be ineffective against *S. epidermidis* biofilms.

AKNOWLEDGMENTS

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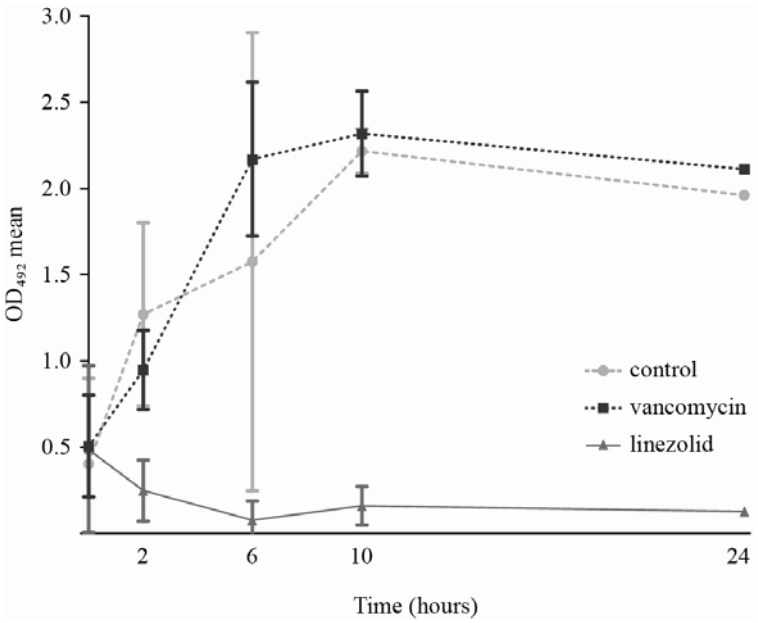

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461 **Fig. 1.** Effect of exposure time to vancomycin or linezolid on biofilms (15 µg/ml). Pre-formed
462 biofilms were exposed to vancomycin or linezolid for different times and their optical density at
463 492 nm was measured in each time. The negative control was not exposed to antibiotics. The error
464 bars represent the confidence interval of 95%, calculated from three independent experiments.

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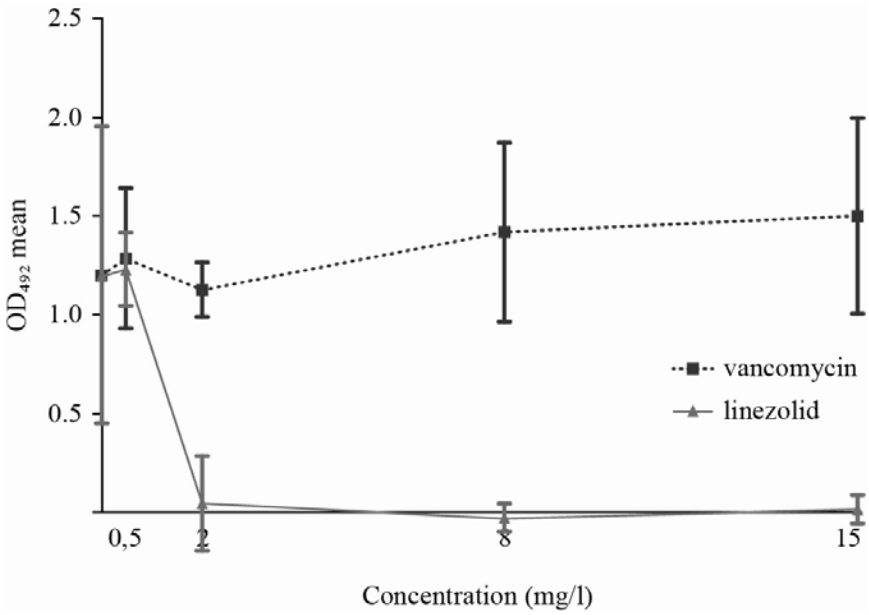

473

474 **Fig. 2.** Effect of concentration of vancomycin or linezolid on biofilms. Pre-formed 24 hours -
475 biofilms were exposed to different concentrations of vancomycin or linezolid for 24 hours and their
476 optical density at 492 nm was measured. The error bars represent the confidence interval of 95%,
477 calculated from three independent experiments.

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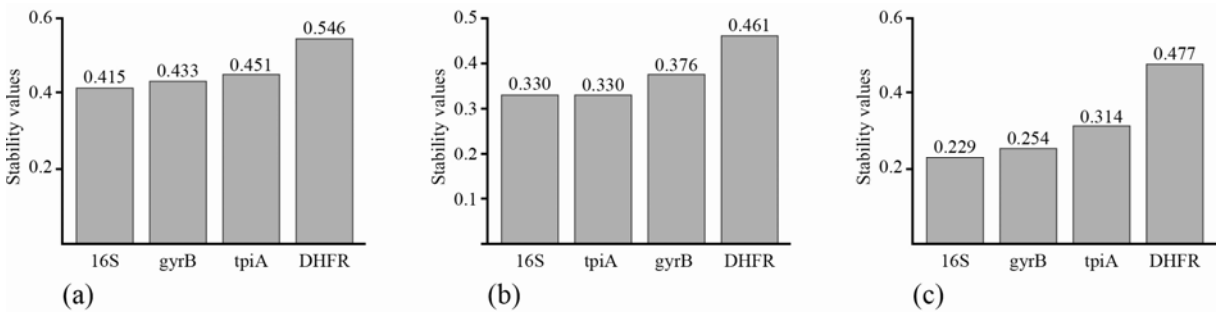

Fig. 3. Stability values of reference genes. **A)** delta Ct method; **B)** geNorm; **C)** Normfinder. Genes with the lowest stability value have the most stable expression.

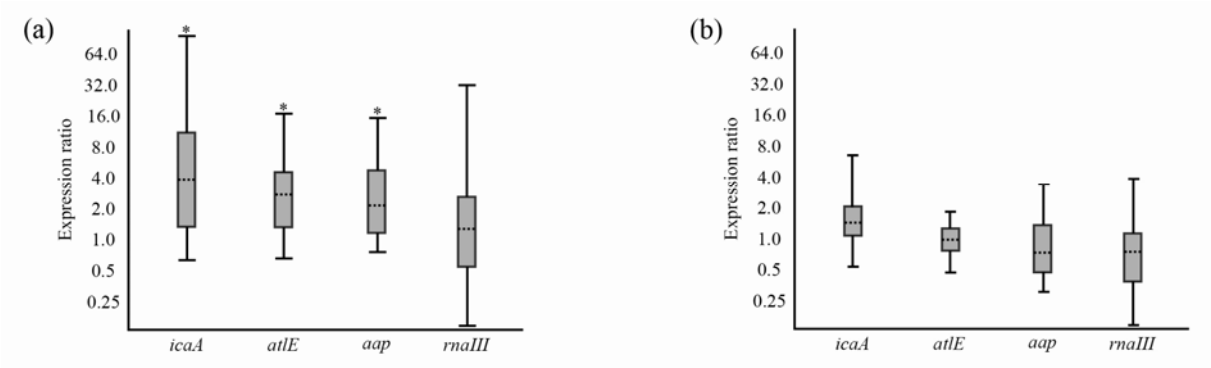

Fig. 4. Relative expression of effector genes *icaA, atlE, aap and rnaIII*. **A)** Expression ratio of linezolid-treated biofilms (15 µg/ml); *icaA* 5.179-fold (p=0.043), *atlE* 3.065-fold (p=0.030), *aap* 2.585-fold (p=0.039), *rnaIII* 1.608-fold (p=0.589); **B)** expression ratio of vancomycin-treated biofilms (15 µg/ml); *icaA* 1.878-fold (p=0.048), *atlE* 1.171-fold (p=0.343), *aap* 0.996-fold (p=0.998), *rnaIII* 0.839-fold (p=0.677). Error bars represent standard error.

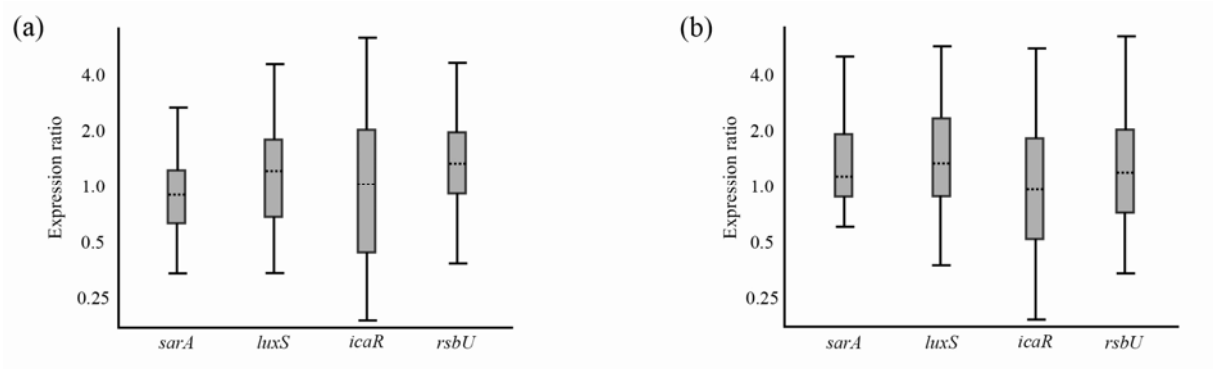

Fig. 5. Relative expression of regulatory genes *sarA, luxS, icaR and rsbU.* **A)** Expression ratio of linezolid-treated biofilms (15 µg/ml); *sarA* 0.814-fold (p=0.411), *luxS* 1.019-fold (p=0.931), *icaR* 0.859-fold (p=0.698), *rsbU* 1.140-fold (p=0.669); **B)** expression ratio of vancomycin-treated biofilms (15 µg/ml); *sarA* 1.233-fold (0.463), *luxS* 1.384-fold (p=0.341), *icaR* 0.971-fold (p=0.944), *rsbU* 1.227-fold (p=0.532). Error bars represent standard error.

Table S1. Oligonucleotides used for qPCR.

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)
aap	aap F	CTGTCCTCAATAATCCCGAAT	106
	aap R	ACATCGCTTTGGATAAAGAGG	
*atl*E	atlE F	TATCGGTTTGCTTTTGTTGG	129
	atlE R	AGGATGGATTGCTGCTAAGG	
DHFR	DHFR F	ATTCGACTTCCCAGTTTTCG	108
	DHFR R	AGGCAATGATTGACCAGGTA	
*ica*A	icaA F	TGGATATTGCCTCTGTCTGG	100
	icaA R	TTATCAATGCCGCAGTTGTC	
*ica*R	icaR F	CTGTCCTCAATAATCCCGAAT	106
	icaR R	ACATCGCTTTGGATAAAGAGG	
*gyr*B	gyrB F	CATCGCCCATTAACATTTCA	117
	gyrB R	TGAACGCAGACCAATTATGG	
*lux*S	luxS F	CATTACAAGCTGGGACTTCG	123
	luxS R	TGGGTTGTCAAACTGGATTC	
*rna*III	rnaIII F	CAATCGGTGATTTAGTAAAATGGA	141
	rnaIII R	GTTGGGATGGCTCAACAACT	
*rsb*U	rsbU F	CGTGCCTCTGTAACACCATC	135
	rsbU R	TGAAGCGTTTGAGGAAATTG	
*sar*A	sarA F	TTGCTTCTGTGATACGGTTGT	106
	sarA R	CGTAATGAACACGATGAAAGAAC	
*tpi*A	tpiA F	TGTAGCGTCAGCAACCTCTT	114
	tpiA R	ATTTGGGCTATCGGTACTGG	
16S	16S F	CCCGTCAATTCCTTTGAGTT	120
	16S R	GCCGTAAACGATGAGTGCTA	

8 CONSIDERAÇÕES FINAIS

Staphylococcus spp utilizam o modo de crescimento em biofilme para iniciar e estabelecer infecções que se tornam recalcitrantes ao tratamento com antibióticos usualmente empregados, mas que são eficazes em testes laboratoriais padronizados, como a concentração inibitória mínima (CIM). Porém, a terapia antimicrobiana falha frequentemente para erradicar essas comunidades multicelulares, o que instiga a pesquisa a encontrar uma justificativa e uma forma eficaz de combater os biofilmes utilizando antibióticos já disponíveis e em concentrações e práticas médicas adequadas ao paciente. Os métodos utilizados para a determinação da formação do biofilme são padronizados e amplamente utilizados em grupos de pesquisa no mundo todo, e confiáveis para avaliar essa condição. Neste contexto, a verificação das concentrações antimicrobianas para inibir e/ou erradicar o biofilme é essencial para guiar uma terapia adequada, uma vez que a prevalência da formação do biofilme em *Staphylococcus* é comprovadamente alta, por este e outros estudos. A formação de biofilme em *S. aureus* e *S. epidermidis* foi bem mais acentuada do que em outros *Staphylococcus*, e estas duas espécies são as mais freqüentes em infecções crônicas no ser humano. Assim, estudos envolvendo estes dois microrganismos serão sempre de importância clínica e epidemiológica.

Antibióticos como vancomicina, tigeciclina e linezolida apresentaram uma tendência de apresentarem atividades superiores frente biofilmes maduros formados por *S. aureus* e *S. epidermidis*, com os métodos fenotípicos utilizados neste estudo. Para observar isso, foi utilizada uma cepa padrão formadora de biofilme (*S. epidermidis* RP62A) para verificar a expressão de genes quando da exposição de biofilmes maduros a antibióticos. Porém, este estudo descreve que antibióticos altamente empregados como vancomicina, um tratamento de escolha para *Staphylococcus*, apresenta um dos piores desempenhos frente ao biofilme, principalmente quando se avalia a progressão da sua atividade ao longo do tempo, e a sua influência na expressão de genes essenciais para a formação e regulação do biofilme. Por outro lado, tigeciclina demonstrou boa atividade contra *S. aureus* nos métodos utilizados, bem como a linezolida para *S. epidermidis*. Além disso, a linezolida influenciou a expressão de genes associados à formação do biofilme, o que podemos sugerir como uma resposta de defesa a concentrações sub-inibitórias deste antibiótico, visto que com o passar do tempo, observou-se que a linezolida foi capaz de erradicar o biofilme.

A determinação da capacidade de formação de biofilme já é uma prática necessária nos laboratórios, uma vez que sua presença está altamente associada com falha terapêutica. Isso se justifica devido à fisiologia e estrutura do biofilme, que impede a ação de certos antibióticos. Porém, mais estudos principalmente moleculares e utilizando a progressão do antibiótico frente ao biofilme com o passar do tempo pode auxiliar e guiar novas e urgentes metodologias de erradicação do biofilme a fim de beneficiar o paciente.

9 ANEXOS

9.1 APROVAÇÕES DOS COMITÉS DE ÉTICA

Complexo Hospitalar Santa Casa de Misericórdia de Porto Alegre

Universidade Federal de Ciências da Saúde de Porto Alegre

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PARECER CONSUBSTANCIADO

Parecer nº 312/010

Protocolo nº 3330/10

Título: ***"Análise molecular de isolados de Staphylococcus spp. provenientes de hemoculturas e dispositivos implantados".***

Pesquisador Responsável: Pedro Alves D'Azevedo

Instituição onde se realizará – Irmandade da Santa Casa de Misericórdia de Porto Alegre.

Data de Entrada: 02/06/2010

II – Objetivos – Objetivo Geral: Avaliar as características moleculares de isolados de *Staphylococcus sp.* coagulase negativa e *S. aureus* obtidos a partir de hemoculturas e pontas de cateter, de pacientes internados na Irmandade Santa Casa de Misericórdia de Porto Alegre – ISCMPA.

Objetivos Específicos:

• Analisar a freqüência de genes responsáveis pela formação de biofilme bacteriano (*ica* operon, *atlE*, *aap*, *hld*) e de genes responsáveis pela transmissibilidade da resistência aos beta-lactâmicos (SCCmec);
• Analisar a freqüência dos grupos *agr* reguladores da produção do biofilme e correlacionar com a presença dos diferentes cassetes genéticos de resistência;
• Analisar e quantificar a expressão dos genes do quorum-sensing (*atlE*, *aap*, *hld*) e do *ica* operon em diferentes fases do crescimento planctônico e séssil (2h, 4h,8h.16h,24h);
• Detecção da concentração inibitória mínima (MIC) para cada isolado antes da exposição a cada antibiótico;
• Comparar isolados resistentes e susceptíveis aos beta-lactâmicos em relação à expressão dos genes do quorum-sensing e do sistema *agr*.

III - Sumário do Projeto

Descrição e caracterização da amostra: Será realizado um estudo transversal, utilizando isolados consecutivos de *Staphylococcus* spp. coagulase negativa e *S. aureus* de hemoculturas e de pontas de cateter. Estes isolados serão obtidos das placas dos meios de cultura, utilizadas no Laboratório Central da ISCMPA, que seriam encaminhadas para autoclavação e posterior descarte. Este estudo tem previsão de coleta de amostras por 2 anos (agosto de 2010 a julho de 2012).

Critérios de inclusão: Isolados de *Staphylococcus* spp. coagulase negativa e *S. aureus* provenientes de hemoculturas e pontas de cateter de pacientes atendidos no ISCMPA.

Critério de exclusão: A exclusão dos isolados ocorre quando existe contaminação d aplaca de meio de cultura ou, para fins de amostragem, não estiverem de acordo com os critérios estabelecidos para cada parte da execução do trabalho.

Adequação das condições - Hospital escola com infra-estrutura adequada para a realização do estudo descrito.

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IV - Comentários:

- Justificativa do uso de placebo – não se aplica.

- Análise de riscos e benefícios – serão coletadas amostras de placas de cultura, não havendo risco para os pacientes que tiveram o material cultivado. Possível benefício pelo conhecimento gerado na pesquisa para aplicação futura em benefício de outros pacientes.

- Adequação do termo de consentimento e forma de obtê-lo – não se aplica TCLE, somente termo de confidencialidade dos dados que está adequado.

- Informação adequada quanto ao financiamento – será financiado por verba de pesquisa do laboratório do pesquisador responsável.

- Outros centros no caso de estudos multicêntricos – não se aplica.

V - Parecer do Relator- -*"Após avaliação do protocolo acima descrito, o presente comitê não encontrou óbices quanto ao desenvolvimento do estudo em nossa Instituição e poderá ser iniciado a partir da data deste parecer".*

VI - Data da Reunião: 06/07/2010.

"Projeto e Termo de Confidencialidade, Aprovados".

Obs.: 1 - O pesquisador responsável deve encaminhar à este CEP, Relatórios de Andamento dos Projetos desenvolvidos na ISCMPA, Relatórios Parciais (pesquisas com duração superior à 6 meses), Relatórios Finais (ao término da pesquisa) e os Resultados Obtidos (cópia da publicação).

2 – Para o início do projeto de pesquisa, o investigador deverá apresentar a chefia do serviço (onde será realizada a pesquisa), o Parecer Consubstanciado de aprovação do protocolo pelo Comitê de Ética.

Porto Alegre, 15 de Julho de 2010.

Prof.Dr. Cláudio Teloken
Coordenador do CEP/ISCMPA

CEP – COMITÊ DE ÉTICA EM PESQUISA

Protocolo para apresentação de Projeto de Pesquisa aprovado pelo CEP ISCMPA

Data de recebimento CEP UFCSPA	Número
20/08/2010	024/10

Nome do projeto: Análise molecular de isolados de Stapylococcus spp. provenientes de hemoculturas e dispositivos implantados.

Autores: Pedro Alves d'Azevedo e Keli Cristine Reiter

Protocolo: 3330/10

Parecer: 312/010

Data de Aprovação no CEP ISCMPA: 15/07/2010

Secretaria CEP UFCSPA

Edi C. Puiroinik
Secretária do CEP/UFCSPA

9 ANEXOS

9.2 PRODUÇÃO CIENTÍFICA COMPLEMENTAR

9.2.1 Artigos publicados

Journal of Infection Control, v. 2, 2013.

MALDI-TOF MS performance to identify gram-positive cocci clinical isolates in Porto Alegre, RS, Brazil.

Thiago Galvão da Silva Paim, Keli Cristine Reiter, Caio Fernando de Oliveira e Pedro Alves d'Azevedo

Abstract

Until recently, gram-positive cocci identification has mainly relied on conventional and time-consuming phenotypic methods. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid alternative for bacterial identification and our study aimed to compare the performance of this methodology with the golden standard method for gram-positive cocci identification. *Staphylococcus* spp (n=386), *Enterococcus* sp (n=46), *Streptococcus* spp (n=18) clinical isolates and reference strains were studied. MALDI-TOF methodology yielded agreement identification of 440/450 gram-positive cocci isolates identified by conventional phenotypic method. The species with more disagreements on identification was *S. epidermidis* (n=5). Some *S. haemolyticus* isolates displayed two distinct genus identification at the first acquisition by MALDI-TOF MS. Two *E. gallinarum* were inacceptable identified as *S. faecium* and one streptococci isolate was erroneously identified by MALDI-TOF (*S. gordonii* as *S. mitis/oralis*). MALDI-TOF MS is fast and reliable, and can be implemented in a clinical microbiology laboratory setting for gram-positive cocci identification.

9 ANEXOS

9.2 PRODUÇÃO CIENTÍFICA COMPLEMENTAR

9.2.1 Artigos aceitos para publicação

Aceite de artigo na Revista do Instituto de Medicina Tropical de São Paulo em 19/04/2013 (fator de impacto 1.0)

Evaluation of three different DNA extraction methods in coagulase-negative staphylococci Clinical Isolates

Caio Fernando de Oliveira, Thiago Galvão da Silva Paim, Keli Cristine Reiter, Alexandre Rieger e Pedro Alves d'Azevedo

Abstract

Currently, there are several methods to extract bacterial DNA, which are based on different principles. However, DNA amount and quality obtained by these methods is highly variable and microorganism-dependent. Coagulase negative staphylococci (CoNS) present a thick cell wall that is difficult to lyses and, so far, this study was designed to compare quality and amount of CoNS DNA, extracted by three different techniques: one based in boiling extraction, one involving purification with phenol-chloroform and another using solid phase separation column. DNA amount and quality determinations were performed by spectrophotometry. Extracted DNA was also analyzed on agarose gel electrophoresis and by polymerase chain reaction (PCR). Column method and thermal lyses showed better results regarding DNA quality (mean ratio 260/280 = 1.95) and DNA average concentration ($\bar{x}$ = 1,018.2 ng/µL), respectively. All three methods provided DNA compatible for PCR amplification. DNA quality is important because enables a large number of molecular biology techniques performances, as well as a storage material for a longer period of time. In this sense, the extraction method based on separation column was better for CoNS.

9 ANEXOS

9.2 PRODUÇÃO CIENTÍFICA COMPLEMENTAR

9.2.2 Resumos publicados em Anais de congressos

V-2125

Linezolid Inhibition Of Mature Biofilm In Methicillin-resistant *Staphylococcus epidermidis* Clinical Isolates: Comparison With Other Drugs

K. C. Reiter, B. Villa, T. G. S. Paim, C. F. Oliveira, **P. A. d'Azevedo**;
JFCSPA, Porto Alegre, BRAZIL.

Staphylococcus epidermidis create many barriers to successful antimicrobial therapy by adhering to foreign surfaces, difficulting treatment and leading to further device removal. Moreover, mechanisms of biofilm resistance are multifactorial and vary from one organism to another. We investigated the efficacy of linezolid against indwelling device-related methicillin-resistant *S. epidermidis* (MRSE) embedded in biofilm, and compared with others antimicrobials. Minimal inhibitory concentrations (MICs), minimum biofilm inhibitory concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs) were determinate by microtiter plate method. A total of 38 consecutive *S. epidermidis* isolates were recovered between August 2010 and January 2011, of which 14 were biofilm-producers MRSE from patients with indwelling device-related bacteremia and 13 were biofilm-producers MRSE from patients with indwelling device colonization. High MBIC values were associated with increasing biofilm biomass (linezolid r=0.477; gentamicin r=0.796; rifampicin r=0.634 and vancomycin r=0.771), but no correlation was observed with MBEC values. Linezolid demonstrated better *in vitro* antimicrobial activity than other antimicrobials (MBIC: gentamicin p<0.001, rifampicin p=0.019, vancomycin p=0.008; MBEC: gentamicin p<0.001, rifampicin p=0.002, vancomycin p<0.001). Biofilm growth inhibition is strongly associated with biofilm biomass; however biofilm eradication is not dependent of number of cells. In general, linezolid reached better *in vitro* concentrations to inhibit mature biofilm and, although none antimicrobial provide complete biofilm eradication, linezolid demonstrated to be highly active against biofilm-producers MRSE by inhibiting their growing at reachable concentrations.

Acknowledgments/References: Financial support: CNPq, FAPERGS.

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ESPECTROMETRIA DE MASSAS NA IDENTIFICAÇÃO DE STAPHYLOCOCCUS SPP. NO LABORATÓRIO DE MICROBIOLOGIA CLÍNICO

[illegible]

Universidade Federal de Ciências da Saúde de Porto Alegre

Objetivo: [illegible] Casuística e métodos: [illegible] Resultados e conclusão: [illegible]

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PERFIL ENZIMÁTICO DE RESISTÊNCIA DOS ISOLADOS DE ACINETOBACTER BAUMANNII NO ANO DE 2011

CARVALHO [illegible]

[illegible]

Objetivo: [illegible] Casuística e métodos: [illegible] Resultados e conclusão: [illegible]

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IMPORTÂNCIA DE UM SISTEMA AUTOMATIZADO ATUALIZADO NA ROTINA DE UM LABORATÓRIO DE MICROBIOLOGIA CLÍNICA

[illegible]

[illegible]

Objetivo: [illegible] Casuística e métodos: [illegible] Resultados e conclusão: [illegible]

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PERFIL MICROBIOLÓGICO DAS INFECÇÕES DA CORRENTE SANGUÍNEA DE UM HOSPITAL ESCOLA EM 2009

[illegible]

[illegible]

Objetivo: [illegible] Casuística e métodos: [illegible] Resultados e conclusão: [illegible]