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Caracterização fenotípica e genotípica de *Staphylococcus aureus* com hetero-resistência à vancomicina (hVISA) em isolados de Santa Catarina

Porto Alegre
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LISTA DE ABREVIATURAS

BHI – Brain Heart Infusion

CA-MRSA – *Staphylococcus aureus* resistente à meticilina comunitário

CDC – Center of Diseases Control

CIM – Concentração inibitória mínima

CLSI – Clinical Laboratory and Standars Institute

EUCAST – Comitê Europeu de Teste de Susceptibilidade aos Antimicrobianos

FURB – Fundação Universidade Regional de Blumenau

GRD – Glycopetide resistance detection

HA-MRSA – *Staphylococcus aureus* resistente à meticilina hospitalar

hVISA – *Staphylococcus aureus* com heteroresistência à vancomicina

IRAS – Infecções relacionadas à assistência em saúde

LA-MRSA – *Staphylococcus aureus* resistente à meticilina veterinário

MRSA – *Staphylococcus aureus* resistente à meticilina

Mu3 – Cepa de referência hVISA

Mu50 – Cepa de referência VISA

PAP-AUC – Análise de perfil de populações-área sob a curva

PFGE – Eletroforese de campo pulsado

PBP – Proteína ligadora de penicilina

PVL – Leucocidina de Panton Valentine

SCC*mec* – Cassete cromossômico estafilocócico

VRSA – *Staphylococcus aureus* resistente à vancomicina

VISA – *Staphylococcus aureus* intermediário à vancomicina

VRE – Enterococo resistente à vancomicina

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RESUMO

Staphylococcus aureus é um dos principais agentes de infecções comunitárias e infecções relacionadas à assistência em saúde (IRAS). Em estudo multicêntrico SENTRY, conduzido em hospitais brasileiros entre os anos de 2005 a 2008, *S. aureus* aparece como principal agente de infecção da corrente sanguínea (20,2 %), principal agente das infecções de pele e tecidos moles (28,1 %) e segundo agente mais comum de pneumonia em pacientes hospitalizados (24,9 %). Destes, aproximadamente 30 % eram resistentes à meticilina (MRSA). O objetivo deste estudo foi avaliar a epidemiologia molecular dos MRSA isolados em Santa Catarina, assim como detectar a prevalência de *S. aureus* com hetero-resistência à vancomicina (hVISA). Foram utilizados 124 isolados clínicos de MRSA, obtidos de vários sítios anatômicos, de pacientes atendidos em três hospitais em Florianópolis e um hospital em Blumenau, todos localizados no estado de Santa Catarina, região sul do Brasil. As amostras foram colhidas entre novembro de 2009 e outubro de 2012. Apenas um isolado por paciente foi considerado. O perfil de susceptibilidade aos antimicrobianos foi avaliado pela técnica de disco difusão e determinação da concentração inibitória mínima (CIM) por Etest. Também foram utilizadas quatro metodologias de triagem para hVISA (pré-difusão, ágar screening com 4 µg/mL de vancomicina, macro Etest e Etest GRD). A confirmação do fenótipo hVISA foi realizada através da análise de perfil de populações-área sob a curva (PAP-AUC). O perfil epidemiológico foi obtido através da caracterização dos tipos de cassette cromossômico estafilocócico (SCC*mec*) e da análise da

similaridade genética por eletroforese de campo pulsado (PFGE). Os agentes antimicrobianos que demonstraram menores taxas de resistência foram a tetraciclina (20,02 %), sulfazotrim (20,02 %) e cloranfenicol (12,9 %). Não foi detectada resistência à vancomicina, teicoplanina, daptomicina, linezolida e tigeciclina. O SCC*mec* predominante foi do tipo III (54 %), seguido pelo tipo II (21,8 %). Vinte e seis complexos clonais foram encontrados, mas sem evidência de disseminação clonal. Dos 124 MRSA testados, doze foram confirmados como hVISA por PAP-AUC, com uma prevalência de 9,7 %. Entre as metodologias testadas para triagem, a que apresentou melhor sensibilidade foi o ágar screening com BHI (90,9 %) e o Etest GRD demonstrou a melhor especificidade (97,3%). Trata-se da primeira descrição confirmada de hVISA no Brasil. Apesar da baixa prevalência de MRSA no estado de Santa Catarina, os isolados mostraram uma semelhança com outros estudos brasileiros, com uma predominância de SCC*mec* do tipo III (associado ao ambiente hospitalar) e taxas de resistência similares aos perfis epidemiológicos. Os complexos clonais detectados apresentaram uma grande diversidade de tipos de SCC*mec*. Veirificou-se que não há predomínio de um único clone. Como as cidades de Florianópolis e Blumenau estão separadas por 120 quilômetros e os isolados foram colhidos por um período de três anos, não foram encontrados significativa relação epidemiológica entre os complexos clonais. Com relação ao fenótipo hVISA, concluímos que sua detecção é um desafio para o laboratório de microbiologia clínica e a prevalência deve estar subestimada. É fundamental para a escolha correta do antimicrobiano e a mudança da vancomicina por outro fármaco, como a linezolida ou daptomicina.

ABSTRACT

Staphylococcus aureus is a major agent of community-acquired infections and related health care infections. SENTRY conducted a multicenter study in Brazilian hospitals during 2005 to 2008 and found that *S. aureus* was the main agent of bloodstream infections (20.2%), the main agent of infections of skin and soft tissue (28.1%) and the second most common agent of pneumonia in hospitalized patients (24.9%). Of these, approximately 30% were resistant to methicillin (MRSA). The aim of the study was to assess the epidemiology of MRSA isolated in Santa Catarina and to detect the prevalence of *S. aureus* with hetero-resistance to vancomycin (hVISA). We used 124 clinical isolates of MRSA obtained from various anatomical sites from patients in three hospitals in Florianópolis and a hospital in Blumenau, all located in the state of Santa Catarina in southern Brazil. Samples were collected from November 2009 to October 2012. One isolate per patient was evaluated. The profile of antimicrobial susceptibility was evaluated by disc diffusion technique and determination of minimum inhibitory concentration (MIC) by Etest, as well as screening for hVISA by four techniques (pre-diffusion, agar screening, macromethod Etest and Etest GRD). Confirmation of hVISA phenotype was performed by population analysis profile-area under the curve (PAP-AUC). The epidemiologic profile was traced through the characterization of SCC*mec* types and evaluation of genetic similarity by pulsed field gel electrophoresis (PFGE). The antimicrobial agents that demonstrated lower rates of resistance were tetracycline (20.2%), sulfamethoxazole-trimethoprim (20.2%) and

chloramphenicol (12.9%). We did not detect resistance to glycopeptides, daptomycin, linezolid and tigecycline. The predominant type was SCC*mec* type III (54%), followed by type II (21.8%). 26 clonal complexes were detected without evidence of clonal spread. Of the 124 MRSA isolates tested, 12 were confirmed as hVISA by PAP-AUC, a prevalence of 9.7%. Among the methodologies tested for screening, which showed better sensitivity was agar screening with BHI (90.9%) and the Etest GRD showed the best specificity (97.3%). This study is the first description of hVISA in Brazil. Despite the low prevalence of MRSA in the state of Santa Catarina, the isolates were consistent with other Brazilian studies, with a predominance of SCC*mec* type III and rates of antimicrobial resistance similar to the epidemiological profile. The clonal complexes had a wide variety of mobile genetic elements, and there was no substantial prevalence of a particular clone. As the cities of Florianopolis and Blumenau are separated by approximately 120 kilometers and the isolates were collected during a period of three years, there was no significant epidemiological relationship between clonal complexes. We conclude that the detection of hVISA isolates remains a challenge for clinical microbiology laboratories, and the prevalence of hVISA may be underestimated. This finding is essential to select the correct antibiotic therapy and replace vancomycin with a different drug such as linezolid or daptomycin.

I.INTRODUÇÃO

I.1 *Staphylococcus aureus*

Staphylococcus aureus é um coco Gram positivo, encontrado naturalmente na biota de diversas partes do corpo humano, como fossas nasais, faringe, intestinos e pele (SANTOS et al., 2009). Descoberto em 1880 por Sir Alexander Ogston (ROSENBACH, 1884), vem sendo considerado uma grande ameaça à saúde pública desde então, principalmente após o surgimento de resistência aos principais antimicrobianos usados clinicamente para combater infecções causadas por essas bactérias.

Ao longo da história da medicina, *S. aureus* se tornou um dos mais relevantes agentes bacterianos. Desde o início da antibioticoterapia e no decorrer das décadas seguintes, a abordagem terapêutica frente a tal bactéria se consolidou como um dos grandes desafios da medicina. Os índices referentes à prevalência de infecções relacionadas à assistência em saúde (IRAS) causadas por S. *aureus* têm tido notável crescimento devido à grande capacidade, da bactéria, de adquirir resistência aos antimicrobianos mais utilizados em ambiente hospitalar (BARBER, 1961). Ademais, tem-se revelado um aumento no número de casos de infecções por *S. aureus* na comunidade nos últimos vinte anos (LAUTENBACH et al., 2010), demonstrando que a disseminação da resistência não está restrita ao ambiente hospitalar.

I.2 *Staphylococcus aureus* resistente à meticilina (MRSA)

Com a descoberta da penicilina, acreditava-se que a mortalidade por processos infecciosos não seria mais um problema. Apenas um ano após sua industrialização, já apareciam os primeiros isolados de *S. aureus* produtores de penicilinases (BARBER, 1965; LOWY, 2003). Para tentar contornar a situação, foram desenvolvidos derivados semi-sintéticos da penicilina, resistentes à ação das beta-lactamases: a oxacilina e meticilina. A partir da década de 60 identificaram-se *S. aureus* resistentes à meticilina (MRSA) (BARBER, 1965), implicando um grande desafio terapêutico para o tratamento das doenças envolvidas com esse micro-organismo.

Os antimicrobianos beta-lactâmicos tem como sítios de ligação proteínas existentes na parede celular das células bacterianas, as proteínas ligadoras de penicilinas (PBP). Com o passar do tempo e com o uso crescente deste grupo de antimicrobianos (CHAMBERS, 2001), *S. aureus* desenvolveu um mecanismo de resistência, promovendo a alteração estrutural destas PBPs, resultando numa proteína alterada chamada PBP 2a, com baixa afinidade de ligação aos antimicrobianos (APPELBAUM, 2007).

O elemento genético responsável pela produção de PBPs alteradas é o gene *mec*A. A presença deste gene implica na resistência a todos os antimicrobianos beta-lactâmicos (exceto as cefalosporinas de quinta geração) e, dependendo do tipo de cassete cromossômico estafilocócico (SCC*mec*), às outras classes de antibacterianos (HIRAMATSU et al., 1997).

S. aureus pode adquirir resistência a praticamente todas as classes de antimicrobianos utilizadas. Alguns fenótipos ainda são incomuns, como a resistência à linezolida e daptomicina, além de *S. aureus* resistente à

vancomicina (VRSA). Os mecanismos de resistência às classes não β-lactâmicas são variadas e usualmente relacionados à mutação (ácido fusídico, quinolonas, sulfas, oxazolidinonas), bomba de efluxo (tetraciclinas) ou modificação enzimática (aminoglicosídeos, macrolídeos, lincosamidas), demonstrados na Figura 1. Atualmente as taxas de resistência tem aumentado, tornando cada vez mais escassas as opções terapêuticas (JONES et al., 2013).

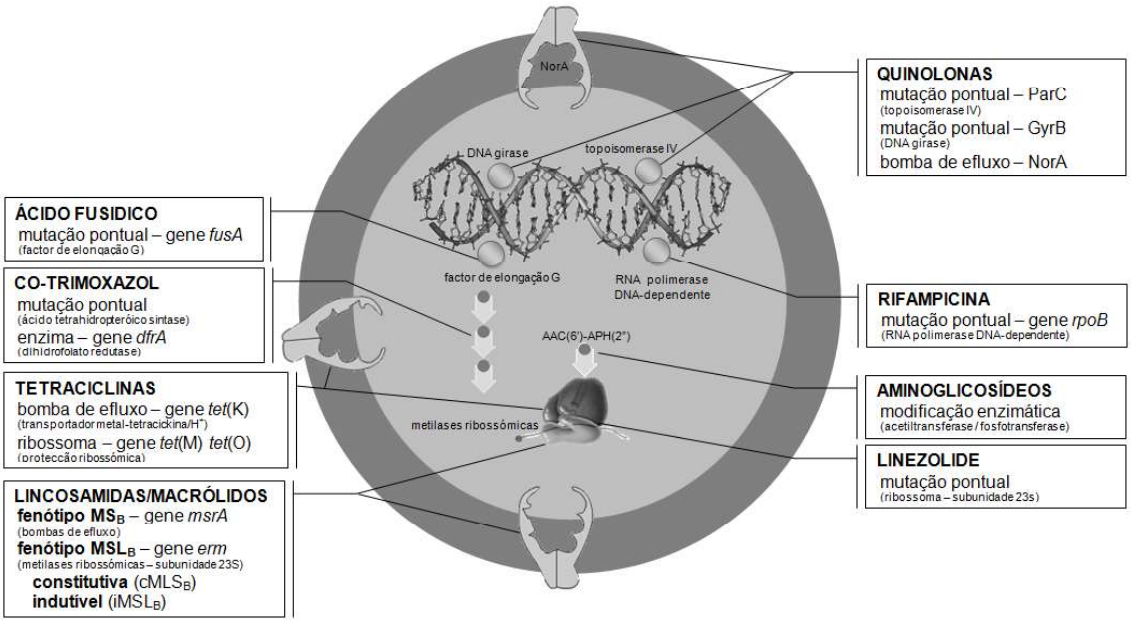


Figura 1 – Mecanismos de resistência de *S. aureus* aos antimicrobianos (MENDES, 2010).

Recentemente foi descrito o primeiro *S. aureus* resistente à vancomicina (VRSA) brasileiro (ROSSI et al., 2014), com presença do gene *van*A. Foi isolado de um homem de 35 anos, diabético, que foi admitido no hospital devido à um quadro depressivo, em novembro de 2011. Desenvolveu um quadro de celulite na perna, sendo tratado com cefalexina oral e gentamicina por via tópica. Recebeu alta, com prescrição de clindamicina. Foi readmitido em junho de 2012, devido ao agravamento dos sintomas psiquiátricos e

infecções recorrentes de pele e tecidos moles, sendo administrado vancomicina e cefepime. A hemocultura foi negativa. Em julho, apresentou febre, senso iniciado tratamento com vancomicina e piperacilina/ tazobactam. Houve crescimento de MRSA na hemocultura, com susceptibilidade à vancomicina, linezolida e clindamicina. Foi iniciado tratamento com teicoplanina por 14 dias. Em agosto, um dia após o término do uso da vancomicina, apresentou quadro febril. Foi isolado *S. aureus* na hemocultura, resistente à vancomicina (32 µg/mL) e teicoplanina (≥ 32 µg/mL), com resistência à clindamicina, sulfazotrim, eritromicina, ciprofloxacin e gentamicina. Foi iniciado tratamento com daptomicina. Em cultura de vigilância, foi isolado *Enterococcus faecalis* resistente à vancomicina. Posteriormente o paciente desenvolveu infecções sistêmicas por *Stenotrophomonas maltophilia* e *Candida albicans*, infecção respiratória causada por *Klebsiella pneumoniae* produtora de ESBL e *Acinetobacter baumannii* resistente aos carbapenêmicos. Foi a óbito em novembro de 2012, utilizando polimixina B, meropenem, linezolida e anfotericina B.

Além de impactar a abordagem terapêutica, infecções bacterianas influenciam diretamente nos custos totais de tratamento. A antibioticoterapia corresponde entre 20% a 50% dos gastos relacionados ao tratamento medicamentoso em ambiente hospitalar (LOURO et al., 2007). Em estudo realizado em 2008 em um Hospital Universitário no estado do Sergipe (Brasil), com 237 pacientes pós-operatórios, os custos totais do tratamento resultaram no montante de R$33.545,49, sendo a quantia correspondente ao tratamento antibiótico de R$21.395,86, ou seja, 64% do total (SANTANA et al., 2014). Se esse valor já se revela elevado contemplando apenas pacientes recém-

operados, quando se infere que há aplicação antibiótica em outras situações além da supracitada, há forte tendência de que o custeio do tratamento se amplie sobremaneira. Uma inoportuna infecção por MRSA, portanto, causaria impacto relevante no orçamento hospitalar.

I.3 Cassete cromossômico estafilocócico (SCC*mec*)

SCC*mec* é constituído basicamente pela presença de repetições terminais diretas e invertidas, dois elementos genéticos essenciais (*mec* e *ccr*) e as regiões *junkyard*. As repetições terminais que determinam a ligação no genoma bacteriano, especificado pela complementariedade das bases. O complexo *mec* é formado por um elemento de inserção IS431*mec*, *mec*A e genes regulatórios *mec*R1 e *mec*I. O complexo *ccr* codifica para as recombinases, que irão mediar a integração ou excisão do complexo SCC*mec* e por isso são responsáveis pela mobilidade do elemento. O restante do elemento compreende as regiões junkyard, localizadas esquematicamente entre e ao redor dos genes *mec* e *ccr*, podendo conter genes que não são fundamentais para a célula bacteriana, mas algumas vezes podem incluir genes de resistência aos fármacos não β-lactâmicos e metais pesados (IWG-SCC, 2009).

A classificação dos tipos de SCC*mec* está relacionado aos tipos de ccr e mec contidos no elemento genético móvel (Figura 2).

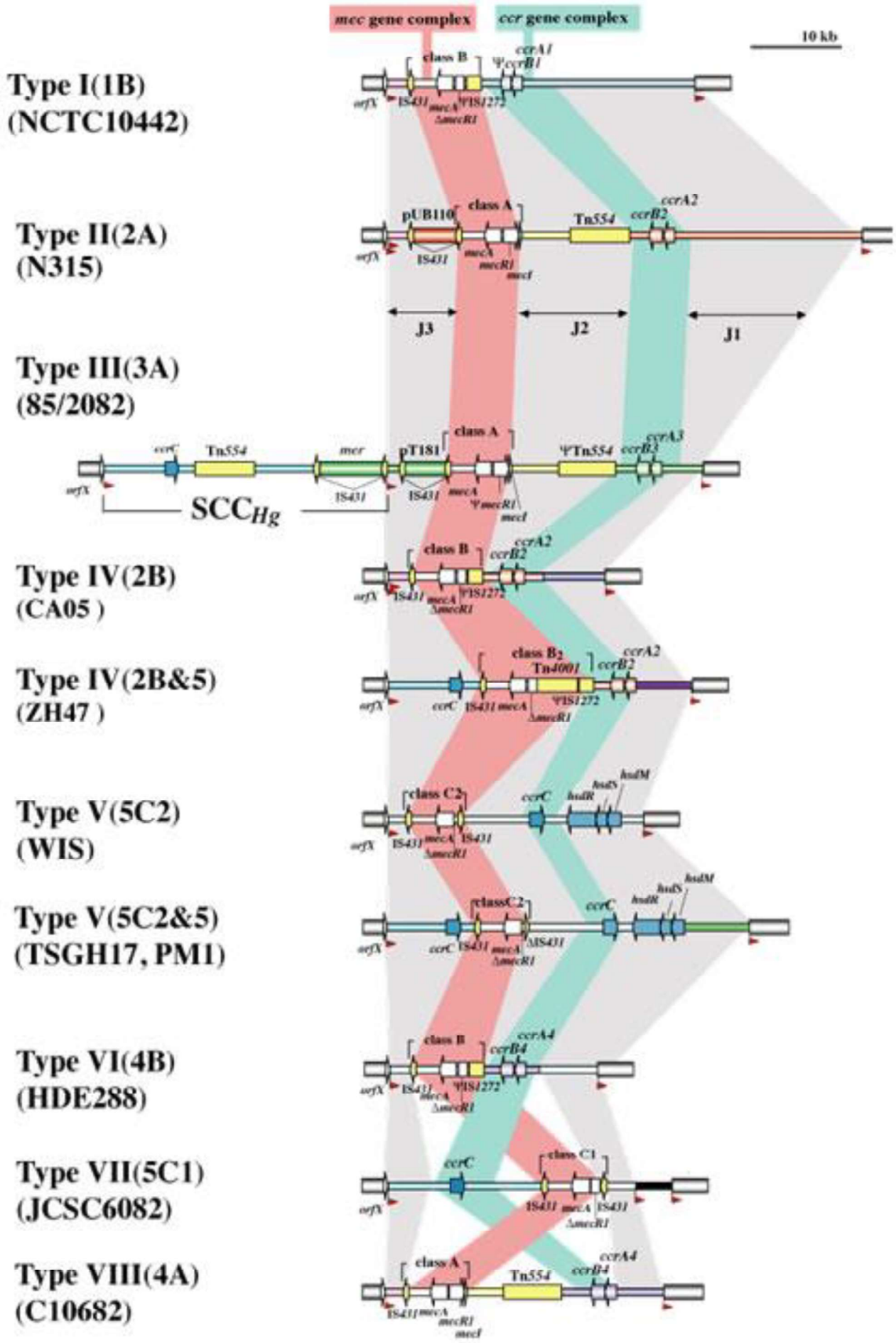

mec gene complex
ccr gene complex
10 kb
Type I(1B)
(NCTC10442)
Type II(2A)
(N315)
Type III(3A)
(85/2082)
Type IV(2B)
(CA05)
Type IV(2B&5)
(ZH47)
Type V(5C2)
(WIS)
Type V(5C2&5)
(TSGH17, PM1)
Type VI(4B)
(HDE288)
Type VII(5C1)
(JCSC6082)
Type VIII(4A)
(C10682)
J3
J2
J1
SCCHg

Figura 2 – Esquema da estrutura genética dos elementos SCC*mec* (IWG-SCC, 2009).
Type I (1B) – *mec* classe B, *ccr* do tipo 1
Type II (2A) – *mec* classe A, *ccr* do tipo 2
Type III (3A) – *mec* classe A, *ccr* do tipo 3
Type IV (2B) – *mec* classe B, *ccr* do tipo 2
Type IV (2B&5) – *mec* classe B, *ccr* do tipo 2 e do tipo 5
Type V (5C2) – *mec* da classe C2, *ccr* do tipo 5
Type V (5C2&5) – *mec* da classe C2, *ccr* do tipo 2 e do tipo 5
Type VI (4B) – *mec* da classe B, *ccr* do tipo 4
Type VII (5C1) – *mec* da classe C1, *ccr* do tipo 5
Type VIII (4A) – *mec* da classe A, *ccr* do tipo 4

Recentemente, foram descritos três novos tipos de SCC*mec*. O complexo IX, que contêm *mec* do tipo C2 e *ccr* do tipo 1. O complexo X é formado por *mec* do tipo C1 e *ccr* do tipo 7 (SHORE; COLEMAN, 2013).

O SCC*mec* do tipo XI, também conhecido como *mec*C foi descrito inicialmente em 2007 e reportado em 2011, em isolado de *S. aureus* de mastite bovina na Inglaterra (GARCIA-ÁLVAREZ; WEBB; HOLMES, 2011). É constituído pelo *mec* do tipo E e *ccr* do tipo 8. Posteriormente foram descritos, além de amostras veterinárias, isolados oriundos de infecções em humanos. Ainda não se conhece muito bem a origem deste elemento, mas se reconhece como prioritária sua detecção devido à sua capacidade de disseminação (PATERSON; HARRISON; HOLMES, 2014).

I.4 Epidemiologia molecular

MRSA podem ser classificados de acordo com sua origem: hospitalar ou comunitário. Para serem considerados comunitários, segundo os critérios do *Center of Diseases Control* (CDC), devem ser isolados de pacientes que não estejam internados ou nas primeiras 48 horas de internação, não apresentam

histórico de infecção ou colonização por MRSA, não relatam internação ou procedimento cirúrgico no último ano e não ter utilizado dispositivos invasivos (CHAMBERS, 2001).

Os isolados comunitários (CA-MRSA) foram descritos na década de noventa. Estão normalmente associados às infecções de pele, muitas vezes em indivíduos jovens e saudáveis, que apresentam lesões de pele ou higiene precária. Usualmente, CA-MRSA apresenta resistência apenas aos agentes β-lactâmicos, sem resistência às outras classes de antimicrobianos. SCC*mec* do tipo IV está mais associado às infeccões comunitárias e, em muitos casos, com produção de leucocidina de Panton-Valentine (PVL) (YAMAKI et al., 2011).

HA-MRSA, por sua vez, são mais prevalentes em pacientes com doenças graves, hospitalização prolongada e/ou uso prévio de antimicrobianos. São mais comuns em idosos, neonatos, imunocomprometidos e pacientes sob diálise, pós cirurgia ou sob uso de dispositivos invasivos. Normalmente apresentam multi-resistência. Como caratcerística molecular, geralmente apresentam SCC*mec* dos tipos I, II ou III (SHORE; COLEMAN, 2013).

As taxas de isolamento de MRSA tem aumentado rapidamente nos últimos anos e tem se tornado endêmicos em muitos países. Clones de CA-MRSA, como USA 300, tem se disseminado na comunidade e também já encontrados associados às IRAS em vários locais do mundo. Mais recentemente, MRSA associados aos animais de produção (LA-MRSA) foram descritos em locais onde trabalhadores rurais acabam entrando em contato com animais infectados (STEFANI et al., 2012).

Em virtude da disseminação de CA-MRSA em ambiente hospitalar e do isolamento de HA-MRSA em pacientes da comunidade, atualmente a

classificação clínica e/ou fenotípica dos MRSA é muito difícil. Torna-se necessário verificar o tipo de SCC*mec*, associado às informações epidemiológicas do paciente, para a correta cartacterização destes isolados (SHORE; COLEMAN, 2013).

I.5 Susceptibilidade de *S. aureus* à vancomicina

A vancomicina é um glicopeptídeo conhecido desde 1956, quando foi colocado à disposição da medicina graças aos trabalhos de Mc Cormick (MCCORMICK et al., 1956). Entretanto foi deixada à parte justamente por causa do sucesso da meticilina, da oxacilina e de outras isoxazolilpenicilinas. A vancomicina é eficiente no tratamento das infecções causadas pelas cepas MRSA, apesar de apresentar efeitos nefrotóxicos e ototóxicos (SRINIVASAN et al., 2002). Em 1997, Hiramatsu, no Japão, descreveu o primeiro isolado de *S. aureus* com resistência à vancomicina (HIRAMATSU et al., 1997).

O mecanismo de resistência de isolados VISA relaciona-se à superprodução de D-Ala D-Ala no peptidoglicano, causando um espessamento da parede celular, que passa a atuar como uma esponja, absorvendo a vancomicina antes que ela alcance seu sítio de ação, favorecendo assim, a uma resposta clínica pobre quando do tratamento de infecções causadas por isolados VISA (HOWDEN et al., 2010). Os isolados hVISA contêm uma subpopulação de microrganismos que sofrem uma reduzida destruição pela vancomicina *in vitro* (PITZ, et al., 2011). As CIMs destas hVISA estão dentro dos padrões de sensibilidade, mas tendem para o nível mais elevado, de 2 µg/mL (HEATHER et al., 2010).

Isolados VRSA caracterizam-se fenotipicamente por CIM ≥ 32 µg/mL para a vancomicina. Tal resistência deve-se à aquisição do gene *van*A (importado de *Enterococcus* spp.) sob a regulação de um sistema de transdução de sinal (genes *van*S e *van*R) ativados unicamente na presença da vancomicina. A sua ativação leva à produção de um conjunto de enzimas que sintetizam precursores modificados do peptidoglicano contendo um terminal D-Ala-D-Lac, com menor afinidade para a vancomicina. A replicação ineficiente de plasmídios de *Enterococcus* spp. em MRSA justificam o número limitado de VRSA no mundo (HOWDEN et al., 2010). Microrganismos do gênero *Enterococcus* que apresentaram resistência ao tratamento com vancomicina, demonstraram seis genes individualmente capazes de gerar resistência ao medicamento: *vanA*, *vanB, vanC*, *vanD*, *vanE* e *vanG.* O primeiro caso de *Staphylococcus aureus* resistente à vancomicina possuía o gene *vanA*, adquirido de uma cepa de *Enterococcus* resistente à vancomicina (VRE) (MUSTA et al., 2009).

I.6 *Staphylococcus aureus* com hetero-resistência à vancomicina (hVISA)

I.6.1 Características

Staphylococcus aureus podem adquirir, durante terapia prolongada com glicopeptídeos, um fenótipo de resistência bastante peculiar. Através de pressão seletiva e, aparentemente, sem transferência de material genético, podem sofrer mutações em sistemas gênicos responsáveis pela produção da

parede celular, tornando-a mais espessa e menos suscetível à ação de antimicrobianos (HOWDEN et al., 2008).

A hetero-resistência de *S. aureus* à vancomicina provoca alterações no aspecto macro-morfológico das colônias, que se apresentam com aparência e pigmentação heterogêneas (Figura 3), dando a impressão de contaminação, podendo confundir o microbiologista (SEVERIN et al., 2004). Seu mecanismo de resistência está associado a uma ativação da síntese da parede celular, que eleva a produção de resíduos de mucopeptideo e reduz a quantidade de antimicrobiano que chega ao local de ação (membrana citoplasmática), ocorrendo assim um espessamento da parede celular e consequente aprisionamento do fármaco (RYBAK et al., 2008). Especula se que cepas hVISA, poderiam ser consideradas precursoras de cepas intermediárias à vancomicina (VISA), uma vez que, após prolongada exposição ao antimicrobiano, poderá ocorrer seleção de população homogênea de células, expressando a característica VISA. Tal fenótipo, além de minoritário, é instável (LONGZHU et al., 2006).

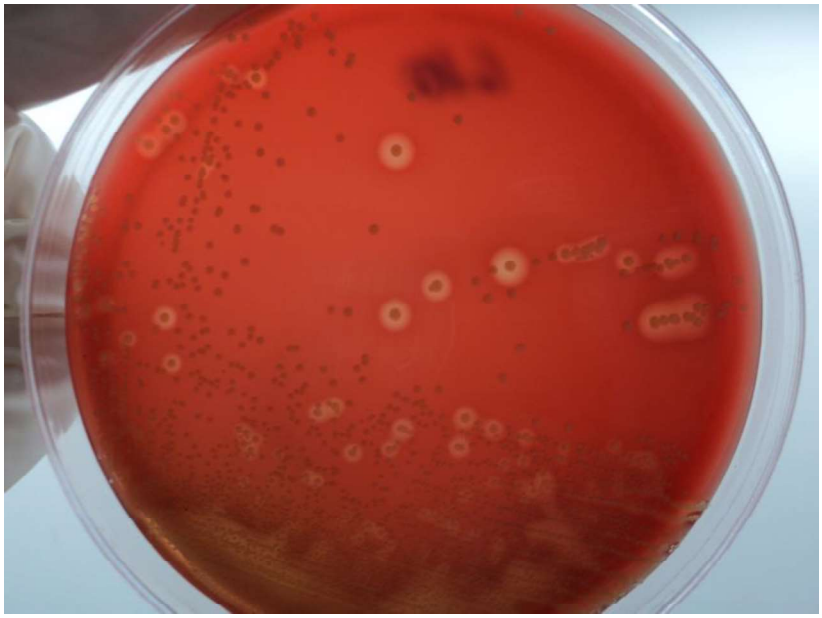


Figura 3 – Isolado clínico hVISA demonstrando variação da morfologia colonial.

Infecções envolvendo hVISA representam um problema único. Tais isolados são suscetíveis *in vitro* à vancomicina (CIM < 4 µg/ml) e, portanto, são classificadas como suscetíveis por métodos laboratoriais padrões, já que contém subpopulações de 1 em 10^6 células que podem crescer na presença de 4 µg/ml de vancomicina (HOWDEN et al., 2010). Especula se que cepas hVISA, poderiam ser consideradas precursoras de cepas VISA, uma vez que, após prolongada exposição ao antimicrobiano, poderá ocorrer seleção de população homogênea de células, expressando a característica VISA (LONGZHU et al., 2006).

Como a detecção de isolados hVISA ainda é um desafio para os laboratórios de microbiologia clínica, sua prevalência pode estar subestimada. Estudos recentes, publicados nos últimos dois anos, mostram taxas bastante variáveis. Na China a taxa entre os MRSA foi de 16,3 % (HU et al., 2013), 11,5 % dos MRSA isolados de corrente sanguínea na Itália forma causadas por hVISA (CAMPANILE et al., 2012). Na Coréia um estudo com 268 MRSA

isolados de bacteremia demonstrou uma prevalência de 37,7 % de (PARK et al., 2012). Já no Taiwan a prevalência foi de 8.1% (LIN et al., 2012). Em estudo no Japão com isolados de corrente sanguínea num período de 21 anos (1987-2007) a taxa de hVISA foi de 18,5 % (TAKATA et al., 2013). Importante destacar que em trabalho realizado em Connecticut, nos Estados Unidos, com 1611 MRSA isolados de amostras clínicas e de culturas de vigilância não foi encontrado nenhum hVISA (FINK et al., 2012).

Os sítios anatômicos mais associados às infecções por hVISA são aqueles que apresentam um maior inóculo bacteriano (abscessos, pneumonia) e outros associados com infecções crônicas (endocardite, osteomiolite), nos quais o uso de vancomicina por períodos prolongados é bastante comum e a baixa penetração do antimicrobiano nesses sítios favorece o desenvolvimento de resistência (SATOLA et al., 2011). Diversos estudos (RYBAK et al., 2008; HORNE et al., 2009; RICHTER et al., 2011; PARK et al., 2012; HU et al., 2013) estabelecem os isolados clínicos de sangue, trato respiratório inferior, feridas de pele, osteomielite e abscessos como os sítios mais comuns de isolamento de hVISA.

Em metanálise publicada em 2011, os índices de falha terapêutica (designada como infecção persistente ou bacteremia) relacionados a isolados hVISA foram 2 vezes mais comuns do que em infecções causadas por *S. aureus* sensíveis à vancomicina (Odds Ratio: 2,37; Intervalo de Confiança 95 %: 1,53-3,67) (VAN HAL; PATERSON, 2012). Dessa forma, um método preciso e prático para a detecção de hVISA nos isolados de laboratório clínico é de importância crescente (LEONARD et al., 2009).

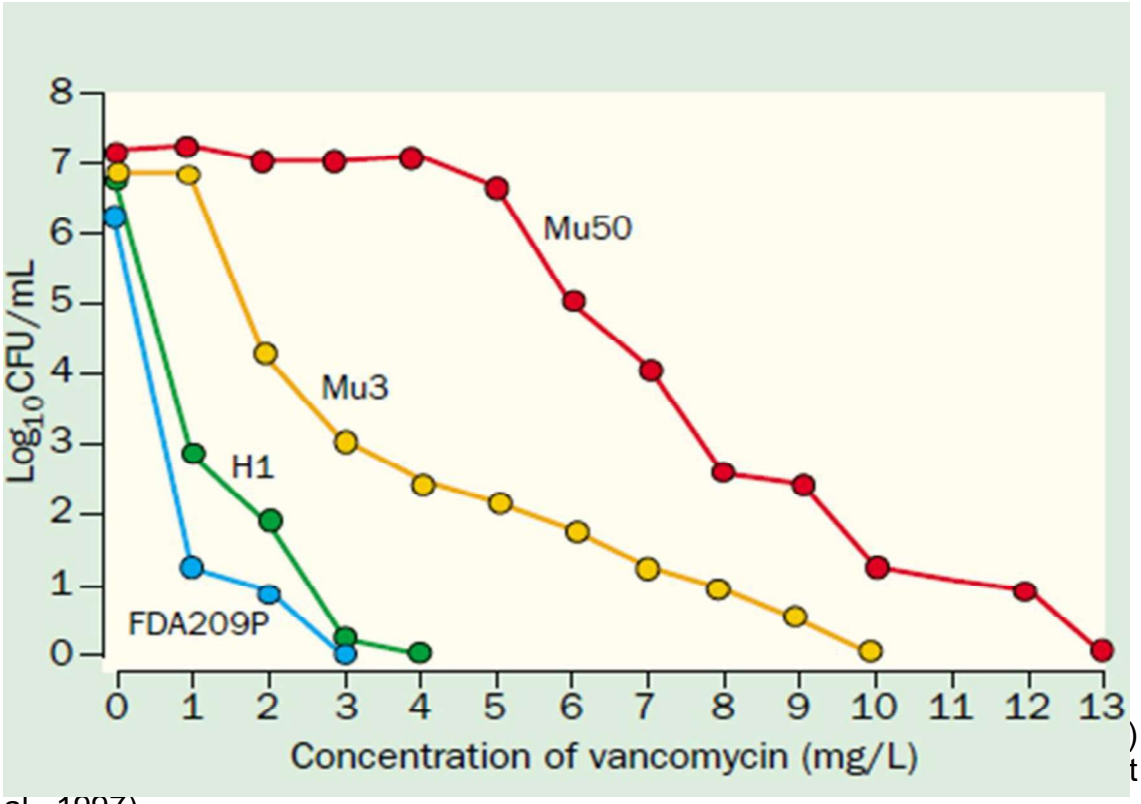
S. aureus com CIMs elevadas (≥ 2 µg/mL) estão entre os principais fatores de risco para o desenvolvimento do fenótipo. Deve-se considerar também que a estratégia laboratorial a ser utilizada depende da metodologia, visto que o Etest tem uma tendência a superestimar as CIMs (PAIVA et al., 2010). Alguns estudos (MUSTA et al., 2009; VAH HAL; JONES, GOSBELL, PATERSON, 2011) demonstram uma relação de hVISA com MICs ≥ 1.5 µg/mL, utilizando Etest. Porém, outros estudos com a mesma metodologia (PARK et al., 2012; CASAPAO et al., 2013) apresentam dados que demonstram associação de hVISA com CIM < 1 µg/mL.

Alguns estudos (RICHTER et al., 2011; PARK et al., 2012) demonstraram baixas taxas de resistências às sulfas em isolados hVISA, com taxas que variam de 9 a 9,9 %. Em contrapartida, estudos com amostras coreanas (GYUNGTAE et al., 2010) e chinesas (HU et al., 2013) mostram valores bem mais elevados, de 58 e 54 %, respectivamente. Já o cloranfenicol demonstrou 47,6 % e, estudo desenvolvido por Hu e colaboradores, em 2013. Já as taxas de resistência aos macrolídeos, lincosamidas, aminoglicosídeos, fluorquinolonas e tetraciclinas estão altas, com valores maiores que 64 %, impossibilitando seu uso empírico (GYUNGTAE et al., 2010; RICHTER et al., 2011; PARK et al., 2012; HU et al., 2013). Dessa forma,o conhecimento do perfil de susceptibilidade local é fundamental para a manutenção de uma terapia empírica adequada.

I.6.2 Detecção laboratorial

Devido ao número crescente de relatos de falhas terapêuticas com o uso de vancomicina e relatos de maus resultados para os pacientes infectados com hVISA, um método preciso e prático para a detecção hVISA nos isolados clínicos é de importância crescente (MANFREDINI et al., 2011). Métodos de referência padronizados para avaliação de susceptibilidade, como a microdiluição em caldo, diluição em ágar, e métodos Etest, não conseguem detectar hVISA, em parte devido ao inóculo ser de pequeno porte, de crescimento relativamente pobre em placas de ágar Mueller-Hinton, ou uma combinação de ambos. O tamanho do inóculo é fundamental para a detecção da subpopulação menor de células resistentes, além disso, cepas hVISA são notoriamente de crescimento lento, com parede celular espessa e características pleomórficas únicas, com colônias de tamanhos variados e nutricionalmente exigentes (PAIVA et al., 2010).

A confirmação de hVISA pelo perfil de análise da população-área sob a curva (PAP-AUC) tem sido a abordagem mais confiável e considerada *gold-standard* (Figura 4). É um método de análise modificado da população que utiliza um protocolo de análise e critérios especificamente projetado para discriminar hVISA e VISA, porém é muito trabalhoso, caro e inadequado para uso de rotina em laboratórios clínicos (YUSOF et al., 2008).



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al., 1997).

Conhecendo os dados epidemiológicos locais e detectando de maneira precisa e precocemente os isolados hVISA, pode se realizar a mudança de agente antimicrobiano, diminuindo as taxas de morbi e mortalidade associadas aos processos infecciosos causados por tais micro-organismos (VAN HAL et al., 2011).

Uma variedade de métodos para a detecção do fenótipo de hetero-resistência foram avaliados com sucesso variável, e nenhum método único de laboratório clínico padronizado foi estabelecido (HOWDEN et al., 2010).

O ágar screening Brain Heart Infusion (BHI) (Figura 5) apresentou uma maior sensibilidade (91 %) e uma boa especificidade (94 %) (VAN HAL et al., 2011) na identificação de hVISA em comparação com outros métodos, utlizando PAP-AUC como método de referência. A sensibilidade de detecção melhorou significativamente com incubação por 48 horas, facilitando a

visualização do crescimento lento dos isolados hVISA. Ressalta-se dessa forma a importância da incubação mais longa, mesmo tendo uma especificidade ligeiramente inferior às outras metodologias utilizadas rotineiramente (SATOLA et al., 2011).

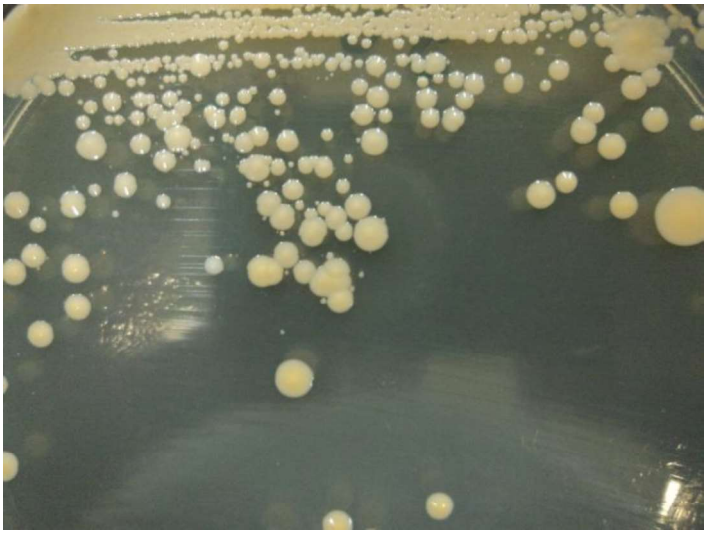

Figura 5 – Isolado clínico hVISA em ágar BHI com 4 µg/mL de vancomicina e 16 g/L de digestão pancreática de caseína. Verificar a variação da pigmentação e tamanho colonial.

O Etest GRD (Glycopeptide Resistance Detection®, AB Biodisk, Sweden) incorpora medidas para melhorar a detecção de hVISA, como o uso de enriquecimento do meio de cultura e a incubação prolongada (LEONARD *et al*., 2009). Usa um gradiente de dupla face de vancomicina e teicoplanina para a detecção de VISA ou hVISA. O resultado da faixa de GRD em combinação com a CIM da vancomicina pode ser utilizado para diferenciar VISA de hVISA (Figura 6). O GRD será considerado positivo quando a determinação de vancomicina e/ou teicoplanina, após 48 horas de incubação, for ≥ 8 µg/mL. No caso de isolados com CIM < 4 µg/mL um teste GRD positivo caracteriza hVISA. Em isolados com CIM > 4 µg/mL o isolado será descrito como VISA

(LEONARD et al., 2009). Apresenta como desvantagem o custo, porém sua praticidade e facilidade de padronização acabam justificando seu uso. Sua sensibilidade pode variar de 57 % a 93 % e especificidade de 82 a 97 %, dependendo do estudo (RICHTER et al., 2011; SATOLA et al., 2011; VAN HAL et al., 2011).

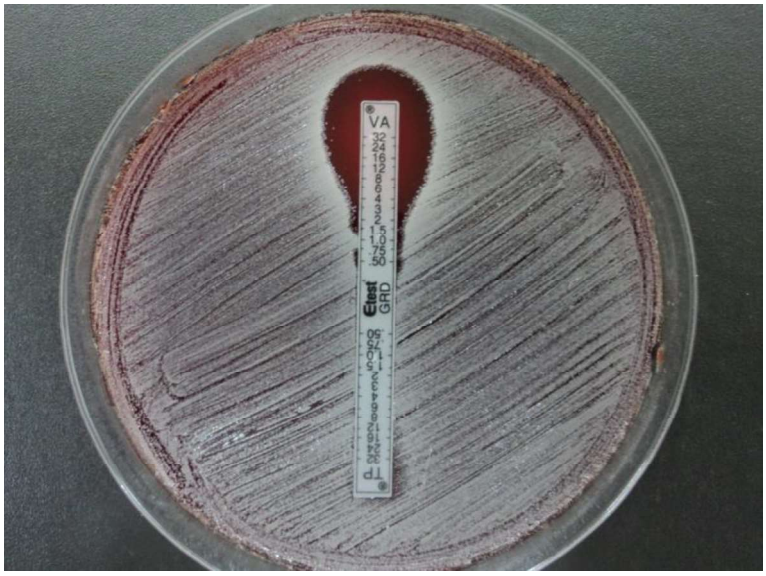

Figura 6 – Isolado clínico hVISA testado com a metodologia do Etest GRD. Verificar que a determinação para teicoplanina foi > 32 µg/mL. A vancomicina apresentou 4 µg/mL.

Já o macro Etest associa as três características fundamentais para detecção de hVISA: inóculo denso, incubação prolongada e meio nutritivo (BHI). É um método simples e rápido, que utiliza tiras de Etest de vancomicina e/ou teicoplanina para avaliar a susceptibilidade aos glicopeptídeos (Figura 7). Os valores de sensibilidade variam de 57 a 89 % e especificidade de 55 a 96 % (SATOLA et al., 2011; RICHTER et al., 2011; VAN HAL et al., 2011).

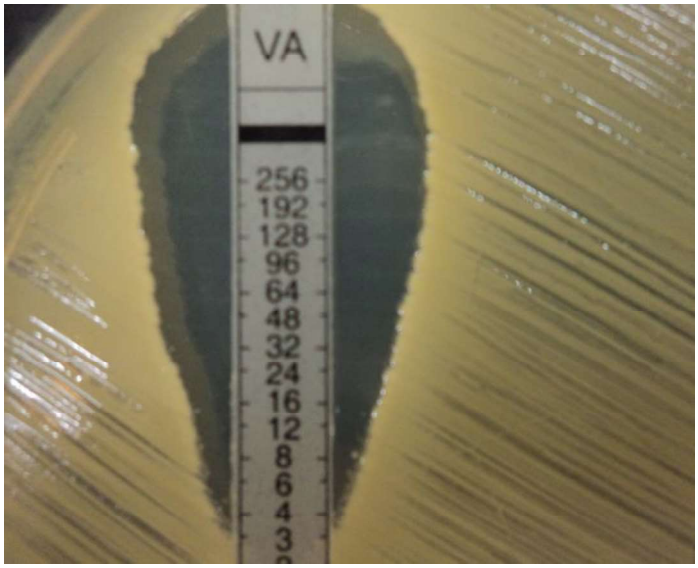

Figura 7 – Meotodologia do macro Etest utilizada como triagem para detecção de hVISA. Verificar a presença de microcolônias dentro do halo de inibição.

Uma metodologia considerada alternativa é a pré-difusão. Consiste em utilizar uma pré-incubação do antibacteriano no meio de cultura, a fim de garantir sua difusão apropriada e homogênea. Após esse tempo, o disco é retirado e a semeadura é feita normalmente, seguindo as técnicas tradicionais. Irá se formar um halo de inibição, que deve ser mensurado e pode ser associado ao fenótipo hVISA (Figura 8).

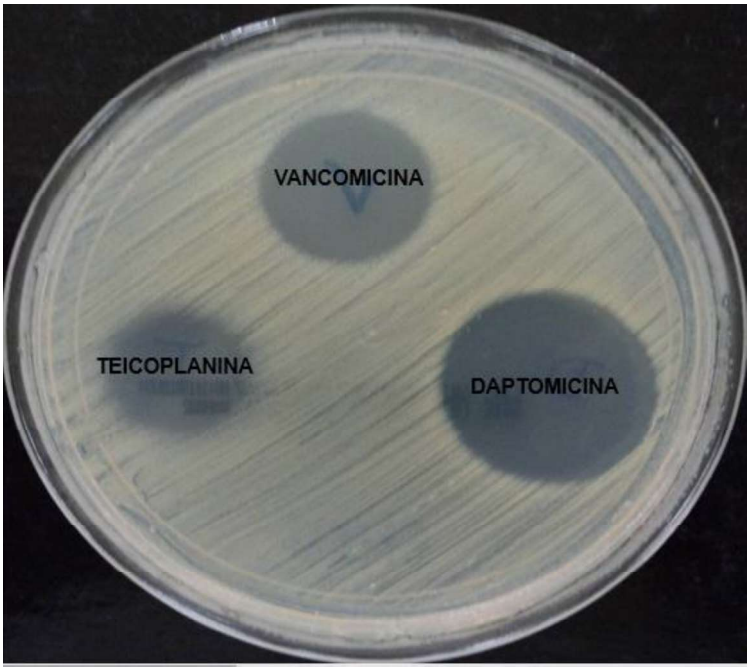

Figura 8 – Metodologia da pré-difusão utilizada em isolado clínico hVISA. Notar que foram testados os antimicrobianos vancomicina, teicoplanina e daptomicina.

Como os testes de triagem apresentam performances irregulares, alguns autores (VAN HAL et al., 2011; HOWDEN et al., 2010) recomendam que sejam combinados, a fim de melhorar a acurácia. Tal estratégia pode esbarrar nos custos (VAN HAL et al., 2011).

I.4.3 Novas abordagens para detecção de hVISA

Como todos os testes descritos apresentam performances variáveis e não ideais, novas abordagens tem sido descritas a fim de uma melhor caracterização dos isolados hVISA.

A produção de δ-hemolisinas quantificadas por expressão gênica (RT-PCR) foi utilizada por Cafiso e colaboradores (2012). Apesar da amostragem reduzida (37 isolados), obtiveram sensibilidade de 89,6 % quando utilizado

ágar sangue de carneiro a 5 % com base de ágar Columbia (CAFISO et al., 2012).

A tecnologia de *single cell genomic*, na qual o genoma de uma única célula bacteriana é amplificado e posteriormente sequenciado completamente. Trata-se de uma ferramenta promissora, pois a partir do momento que se viabilize (através de microscopia de varredura) o isolamento de uma única célula bacteriana com o fenótipo desejado (hVISA), poderemos detectar quais as mutações presentes nessa população minoritária e, dessa forma, inferir com maior propriedade qual os genes envolvidos com o mecanismo de resistência (OWENS, 2012).

Uma ideia bastante interessante foi proposta por Read e Satola (2014). Foram sequenciados todos os doze genes com mutações já descritas em isolados hVISA e desenvolveram um modelo matemático para predizer o fenótipo VISA. Foi disponibilizada uma versão interativa (http://tread.shinyapps.io/VISA-shiny/), na qual o pesquisador pode inserir os dados dos seus isolados (mesmo que não tenham sequenciado todos os genes descritos) e podem ainda relacionar os dados da PAP-AUC ou valores de CIM obtidas por Etest (READ; SATOLA, 2014).

Pesquisadores suíços tem utilizado técnicas de microcalorimetria para detecção de VISA, hVISA e VRSA. Utilizam uma ampola com 3 mL de caldo BHI com concentrações crescentes de vancomicina. Inoculam 100 µL de suspensão bacteriana na escala 2 de McFarland e incubam em equipamento apropriado (microcalorímetro isotérmico). As temperaturas são medidas constantemente e monitoradas por 72 horas. O equipamento tem uma acurácia de detecção de 0,2 °C (ENTENZA et al., 2014). Os resultados foram

discriminatórios, porém um estudo mais amplo que, além de um número amostral maior, necessita de uma maior diversidade genética para considerar a metodologia como eficaz no diagnóstico da diminuição da susceptibilidade à vancomicina.

Também foram utilizados dados do sequenciamento completo de isolados e utilização de ferramentas de bioinformática para avaliação das características genéticas dos isolados hVISA (ALAM et al., 2014). Foram relacionadas as mutações no gene *rpo*B (locus H481) como um importante marcador de aumento na CIM da vancomicina (medida por Etest). Trata-se de uma investigação bastante complexa e cara, que apesar de interessante, dificilmente poderá ser adotada na rotina dos laboratórios de microbiologia clínica

II. JUSTIFICATIVA

A terapêutica antimicrobiana adequada e precoce é fundamental para diminuir os índices de mortalidade relacionados aos processos infecciosos causados por isolados hVISA. A falha terapêutica em infecções causadas por isolados considerados susceptíveis ocorre, principalmente, devido a isolados hetero-resistentes, ou seja, uma subpopulação com sensibilidade diminuída a vancomicina. Dessa forma, um resultado susceptível pode apresentar falha terapêutica quando tratado com glicopeptídeo. A avaliação da tolerância aos glicopeptídeos é importante para prognosticar falhas no tratamento, e pode ser considerado um dos primeiros passos para prevenir e controlar a emergência de resistência à vancomicina em *S. aureus* (HOWDEN et al., 2010).

Como o fenótipo de resistência hVISA manifesta-se de maneira heterogênea e minoritária na população bacteriana (apenas 1 em cada um milhão de micro-organismos apresentam tal característica), os métodos usualmente utilizados em laboratórios de microbiologia clínica (determinação da CIM através de microdiluição ou Etest) falham na sua detecção, sujeitando o paciente à terapêutica com vancomicina com possível falha terapêutica (SATOLA et al., 2011).

Vários estudos vem demonstrando a prevalência de hVISA entre os isolados de *S. aureus*, sendo que as taxas variam de acordo com o País: Austrália (9,4 %), Estados Unidos (8,3 %), Israel (6 %), França (11 %), Turquia (32 %) (HOWDEN et al., 2010). Em metanálise realizada em 2011, os índices de falha terapêutica (designada como infecção persistente ou bacteremia) relacionados a isolados hVISA foram 2 vezes mais comuns do que em

infecções causadas por *S. aureus* sensíveis à vancomicina (OR: 2,37; IC 95 %: 1,53-3,67) (VAN HAL; LODISE; PATERSON, 2012).

Além disso, estudo multicêntrico recente (JONES et al., 2013) demonstrou que as taxas de resistência à meticilina tem aumentado, acarretando assim um maior uso de vancomicina e, com isso, as taxas de diminuição de susceptibilidade aos glicopeptídeos tendem a aumentar.

Dessa forma, o conhecimento do perfil epidemiológico local, associado às informações clínicas do paciente, são imprescindíveis para uma antibioticoterapia empírica adequada e também para mudança no fármaco escolhido caso seja detectado um fenótipo hVISA (YAMAKI et al., 2011).

IV. OBJETIVOS

IV.1 Objetivo geral

- Avaliar a epidemiologia molecular dos MRSA isolados em Santa Catarina de 2009 a 2012

IV.2 Objetivos específicos

- Verificar se houve disseminação clonal de MRSA;
- Avaliar os métodos de detecção de hVISA utilizados rotineiramente em laboratórios de microbiologia clínica;
- Verificar a utilização da metodologia de pré-difusão na triagem para hVISA;
- Descrever a incidência de hVISA em Santa Catarina;
- Analisar o perfil de susceptibilidade aos antimicrobianos e a epidemiologia molecular dos isolados hVISA.

IV. ARTIGO 1.

Artigo publicado no periódico *Diagnostic Microbiology & Infectious Diseases*, v. 79, n. 4, p. 401-404, 2014. Fator de Impacto: 2,568

Is prediffusion test an alternative to improve accuracy in screening hVISA strains and to detect susceptibility to glycopeptides/lipopeptides?

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ABSTRACT

Background: Since 2009, the Clinical and Laboratory Standards Institute (CLSI) no longer recommends disk diffusion to determine vancomycin susceptibility among *Staphylococcus aureus*. Considering that broth microdilution tests are time and labor-intensive, and that Etest® is expensive for developing countries, laboratories are faced with difficulties to correctly detect vancomycin susceptibility. Furthermore, the characterization of heteroresistant vancomycin-intermediate *Staphylococcus aureus* strains (hVISA) is even more challenging, as no routine standardized laboratory methods are available. Among various methodologies proposed, prediffusion appears to be a promising option. **Materials:** A total of 124 *S. aureus* isolates, 112 methicillin-resistant (MRSA) and 12 hVISA recovered from inpatients attended in hospitals of Santa Catarina State, Southern Brazil were evaluated. The minimum inhibitory concentration (MIC) of vancomycin, teicoplanin and daptomycin were determined by Etest® (Biomerieux, Durham, NC) following the manufacturer's instructions. Prediffusion tests using NeoSensitabs® tablets (Rosco Diagnostica, Denmark) were done by placing discs in contact with Mueller Hinton agar plate for 2 hours, removing

the discs and incubating the plates for additional 22 hours at room temperature. Subsequently, plates were inoculated with a 0.5 McFarland bacterial suspension and incubated for 24 hours at 35°C. Inhibition zones were measured and zones ≤ 22 mm indicated reduced susceptibility to vancomycin, < 20 mm to teicoplanin, and < 21 mm to daptomycin. **Results:** All isolates were susceptible to vancomycin (MICs: 0.5 to 3 µg/mL) by Etest®. However, according to prediffusion test, 17 isolates presented reduced susceptibility to vancomycin and of these, 12 were confirmed as hVISA using populational analysis. Prediffusion presented 78.6%, 94.5%, 64.7% and 97.2% of sensitivity, specificity, positive and negative predictive values, respectively. Considering daptomycin, prediffusion results were in agreement with susceptibility data (MICs), as all isolates were susceptible. **Conclusion:** Considering that characterizing hVISA is challenging, and that MIC determination is not adequate to characterize this phenotype, prediffusion test was a viable alternative to screening hVISA and reduced susceptibility to vancomycin. It was simple and low cost, with accuracy comparable to other well-established methods.

INTRODUCTION

Vancomycin is a glycopeptide known since 1955, when it was placed at the disposal of medicine through the work of McCormick (McCormick et al., 1955). Initially, due to the success of methicillin, oxacillin, and other isoxazolepenicillins, it was not often used, though with the emergence of methicillin resistance in the 1960's, came to be used quite often. In 1997, a strain of *Staphylococcus aureus* resistant to vancomycin (vancomycin-resistant *S. aureus* [VRSA]) (Bobin-Dubreux et al., 2001) was identified in Japan. This phenotype had not have a wide dissemination and the

drugs most frequently used to treat infections caused by methicillin-resistant *S. aureus* (MRSA) is still vancomycin, besides the daptomycin and linezolid (Jones, 2006).

Detection of *in vitro* susceptibility of isolates to these drugs is challenging. Since 2009, the Clinical and Laboratory Standards Institute (CLSI) no longer recommends disk diffusion to determine vancomycin susceptibility due to its high molecular weight resulting in decreased diffusion in culture media (Heather et al., 2010).Based on current CLSI recommendations, susceptibility to linezolid and teicoplanin may be determined by disk diffusion method, while vancomycin and daptomycin susceptibilities should be assessed by dilution methods or some specific_diffusion tests in agar. These tests are extremely laborious and expensive, making them difficult to implement, especially in developing countries.

Taking into account the seriousness of MRSA infections, early and aggressive antimicrobial therapy is essential to reduce mortality. Treatment failures in infections caused by susceptible isolates are primarily due to heteroresistant vancomycin-intermediate *Staphylococcus aureus* strains (hVISA), a subpopulation with reduced susceptibility to vancomycin. Thus, *in vitro* susceptibility may not be enough for the physician to achieve treatment success. The evaluation of tolerance to glycopeptide antibiotics is important for predicting treatment failure (Van Hal; Paterson, 2011), and can be considered the first step to preventing and controlling the emergence of vancomycin resistance in *S. aureus* (Howden et al., 2010). As the hVISA resistance phenotype manifests itself heterogeneously and is a minor component of the bacterial population (1 in 10^6 microorganisms) the methods commonly used in clinical microbiology laboratories (MIC determination by Etest or microdilution) fail to detect potential resistance, and vancomycin therapy may fail (Satola et al., 2011).

NeoSensitabs® (Rosco Diagnostica, Taastrup, Denmark) are tablets containing antimicrobial (9 mm diameter and 1.5 mm thick) stable at room temperature, which were developed to evaluate bacterial susceptibility to high molecular weight drugs such as vancomycin, teicoplanin, daptomycin and colistin. The initial prediffusion allows a homogeneous diffusion of the antibiotic in the culture medium, making possible to discriminate strains with reduced susceptibility to these agents. The aim of this study was to evaluate the prediffusion methodology for the detection of decreased susceptibility to glycopeptides and daptomycin as well as its use in the detection of hVISA isolates.

METHODS

Samples

Clinical isolates (n=124) were collected (March 2009 to February 2013) from inpatients attended in three hospitals in Florianópolis (Hospital de Caridade, Hospital Governador Celso Ramos and Cardio SOS) and a hospital in Blumenau (Hospital Santa Isabel), all located in Santa Catarina state, Southern Brazil. One isolate per patient was considered. Identification was done using the following tests: Gram staining, catalase production, mannitol fermentation, coagulase and DNase production. Susceptibility to methicillin was determined by cefoxitin disk diffusion, according to the interpretive criteria of CLSI (2013).

Phenotypic tests for screening and confirmation of heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hVISA)

Three screening tests were used for the detection of hVISA strains. BHI agar plates (Difco, Becton Dickinson and Company) containing 4 µg/mL of vancomycin and

16 g/liter of pancreatic digest of casein were inoculated with a prepared 0.5 McFarland standard inoculum. After 24h of incubation at 35-37°C the growth of more than 20 colonies was considered a positive test for hVISA (Satola et al., 2011). Using Etest® Glycopeptide Resistance Detection (GRD) (Biomerieux, Durham, NC, USA), the isolate was considered hVISA when the MIC for teicoplanin is 12 µg/mL or 8 µg/mL for teicoplanin and vancomycin after 48 hours (Van Hal et al., 2011). The Etest® macromethod (Biomerieux, Durham, NC, USA) was performed using a 2.0 McFarland inoculum on BHI agar plates and readings taken at 24 and 48 hours, where an MIC of 8 µg/mL to vancomycin identified hVISA isolates (Van Hal et al., 2011).

All isolates with at least one positive screening test were confirmed through populations profile analysis (PAP/AUC). The area under the curve (AUC) was calculated using Mu3 (hVISA) as a control strain. For confirmation of a hVISA isolate, the ratio of area under the curve for the isolate divided by that of Mu3 should be greater than or equal to 0.9 (Wootton et al., 2001).

Minimum inhibitory concentration (MIC)

MIC values to vancomycin, teicoplanin and daptomycin were obtained by the Etest® methodology (Biomerieux, Durham, NC). Interpretation was performed, following CLSI (2013) guideline.

Prediffusion tests

Neosensitabs® containing 30 µg vancomycin (VAN), 30 µg teicoplanin (TEI) or 30 µg daptomycin/100 µg calcium (DAPCal) (Rosco Diagnostica, Taastrup, Denmark) and prediffusion tests were performed following the manufacturer's guidelines (Supplement User's Guide, 2006). The tablets were placed on the surface of Mueller

Hinton agar (Biomerieux, Durham, NC, USA). They were inverted and incubated for 2 hours at room temperature. After this period, plates were incubated for further 22 hours to ensure the complete diffusion of antibiotics. Then, plates were inoculated with bacteriaand incubated at 35°C for 24 hours and inhibition evaluated. Isolates with vancomycin ≤ 22 mm and/or teicoplanin < 20 mm inhibition zones were considered VISA/hVISA. For daptomycin, isolates < 22 mm were defined as resistant.

Quality control

To ensure the quality and accuracy of the test results, *Staphylococcus aureus* strains ATCC 29213 (MSSA), ATCC 43300 (MRSA), ATCC 700698 (hVISA) and ATCC 700699 (VISA) were used.

RESULTS

All isolates were susceptible to vancomycin, considering E-test results. MICs were 0.5 µg/mL (0.8 %), 0.75 µg/mL (0.8 %), 1.0 µg/mL (17.7 %), 1.5 µg/mL (42.7 %), 2.0 µg/mL (32.3 %), and 3.0 µg/mL (5.6 %). Considering these data, all isolates would be characterized according to CLSI criteria as susceptible to vancomycin. Seventeen isolates had divergent results for MIC and prediffusion:they were considered susceptible by the CLSI breakpoints. Among the 17 divergent isolates, 2 had MIC of 1.5 µg/mL, 10 presented MIC of 2.0 µg/mL and 5 MIC of 3.0 µg/mL (Figure 1).

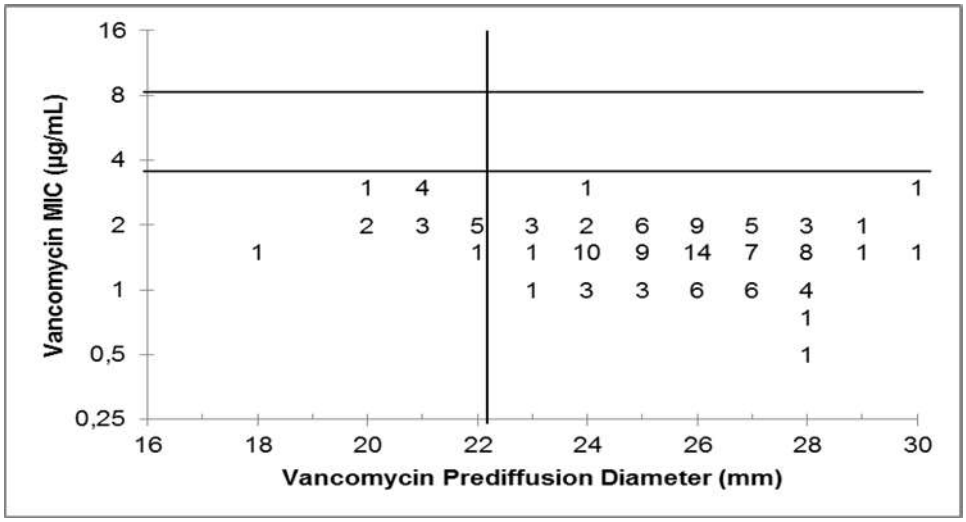

Figure 1 - Vancomycin prediffusion results

Teicoplanin data indicated MICs of 0.19 µg/mL (0.8 %), 0.25 µg/mL (0.8 %), 0.38 µg/mL (1.6 %), 0.5 µg/mL (3.2), 0.75 µg/mL (1.6 %), 1.0 µg/mL (4.8 %), 1.5 µg/mL (17.7 %), 2.0 µg/mL (16.9 %), 3.0 µg/mL (33.9 %), 4.0 µg/mL (13.7 %), 6.0 µg/mL (1.6 %), 8.0 µg/mL (2.4 %), and 12.0 µg/mL (0.8 %). Divergences were observed for 14 isolates, which were classified as intermediate according to prediffusion testing but considered susceptible according to CLSI criteria. Among them, 1 had an MIC of 1.5 µg/mL, 7 at 3.0 µg/mL, 4 at 4.0 µg/mL, and 2 at 8.0 µg/mL.

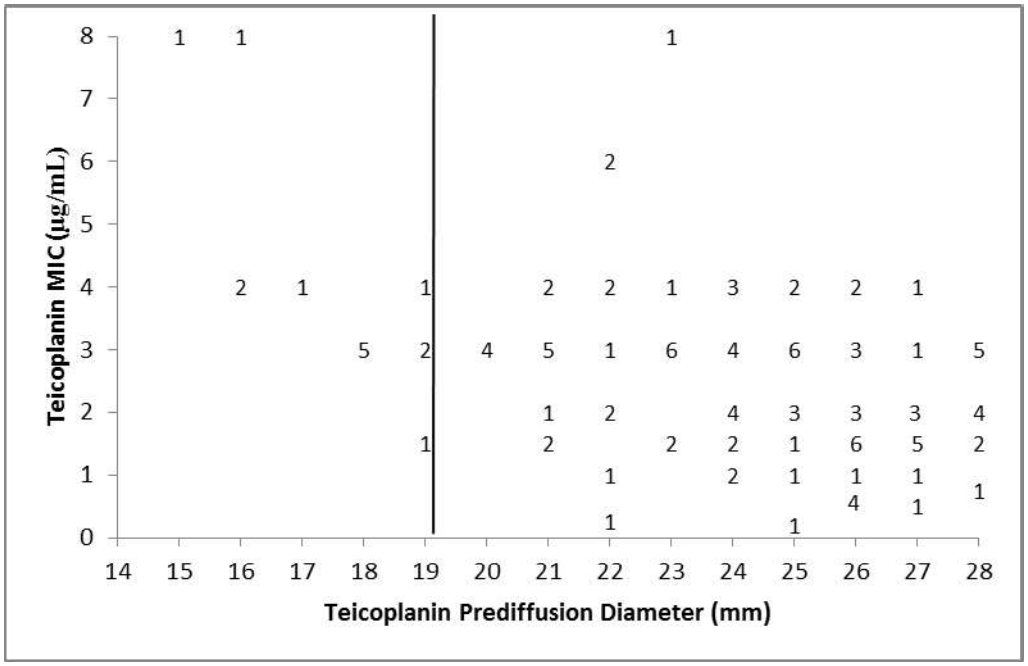

Figure 2 - Teicoplanin prediffusion results

Vancomycin and teicoplanin susceptibility data demonstrated that 21 clinical isolates were intermediately susceptible to vancomycin and/or teicoplanin, which indicated these isolates were VISA using the prediffusion method.

All isolates were submitted to screening tests for the hVISA phenotype (GRD, Etest® macromethod and agar screening with vancomycin), and of those that tested positive for any of the three screening tests, the phenotype was confirmed by PAP-AUC. Of the 124 isolates, 12 (9.7%) were characterized as hVISA (Table 1).

Table 1 - hVISA detection by the prediffusion method compared with the reference test (PAP-AUC)

	hVISA	Non-hVISA	TOTAL
Positive prediffusion	11	10	21
Negative prediffusion	1	102	103
TOTAL	12	112	124

Based on the above data, 91.7% sensitivity, 83.1% specificity, a positive predictive value of 52.4%, a negative predictive value of 97.1% and an accuracy of 89.5% were established for prediffusion test.

Table 2
Results showed that a disagreement between the prediffusion and screening tests for hVISA.

Isolate	Vancomycin (mm)	Teicoplanin (mm)	Etest GRD vancomycin/teicoplanin (μg/mL)	Agar screening	Etest macromethod (μg/mL)	PAP-AUC	Interpretation
SI1	21	21	1.5/4	No	8	0.8	False positive
SI5	22	18	2/6	No	8	0.77	False positive
SI9	22	19	1/1.5	No	6	0.72	False positive
SI10	21	17	1.5/6	No	8	0.86	False positive
SI12	21	16	2/8	Yes	12	0.89	False positive
SI26	22	18	1.5/8	Yes	6	0.78	False positive
SI29	21	19	3/8	Yes	8	0.84	False positive
L4	25	18	1.5/2	Yes	4	0.63	False positive
L10	23	20	2/8	Yes	4	0.92	False negative
L64	21	20	1.5/4	No	8	0.79	False positive
L83	24	19	1/4	No	3	0.71	False positive

Daptomycin data indicated MIC values of 0.125 µg/ml (3.2 %), 0.19 µg/mL (5.6 %), 0.25 µg/mL (8.9 %), 0.38 µg/mL (23.4 %), 0.5 µg/mL (33.9 %), 0.75 µg/mL (21 %) and 1.0 µg/mL (4 %). All isolates presented, therefore, a susceptible phenotype.

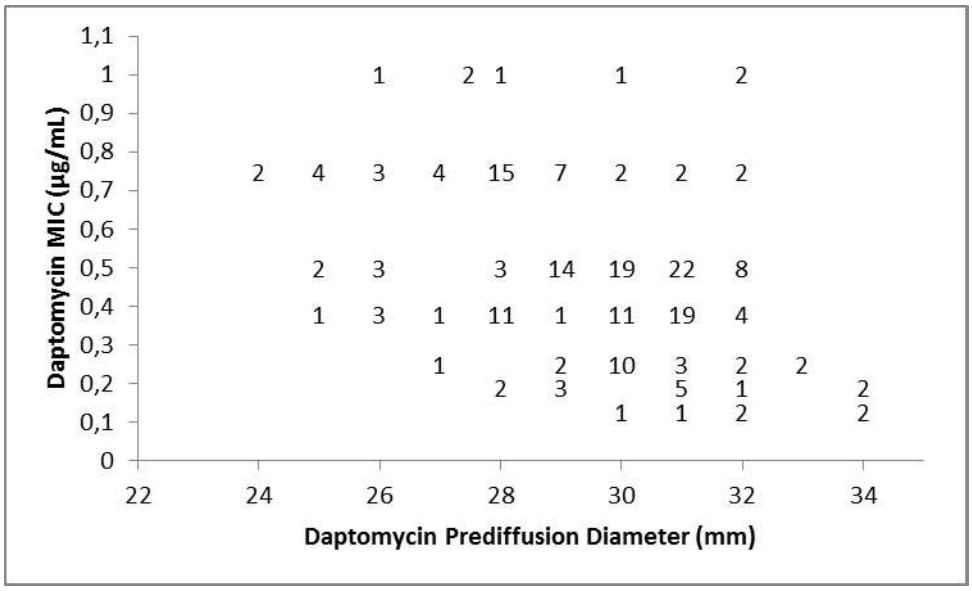

Figure 3 – Daptomycin prediffusion results.

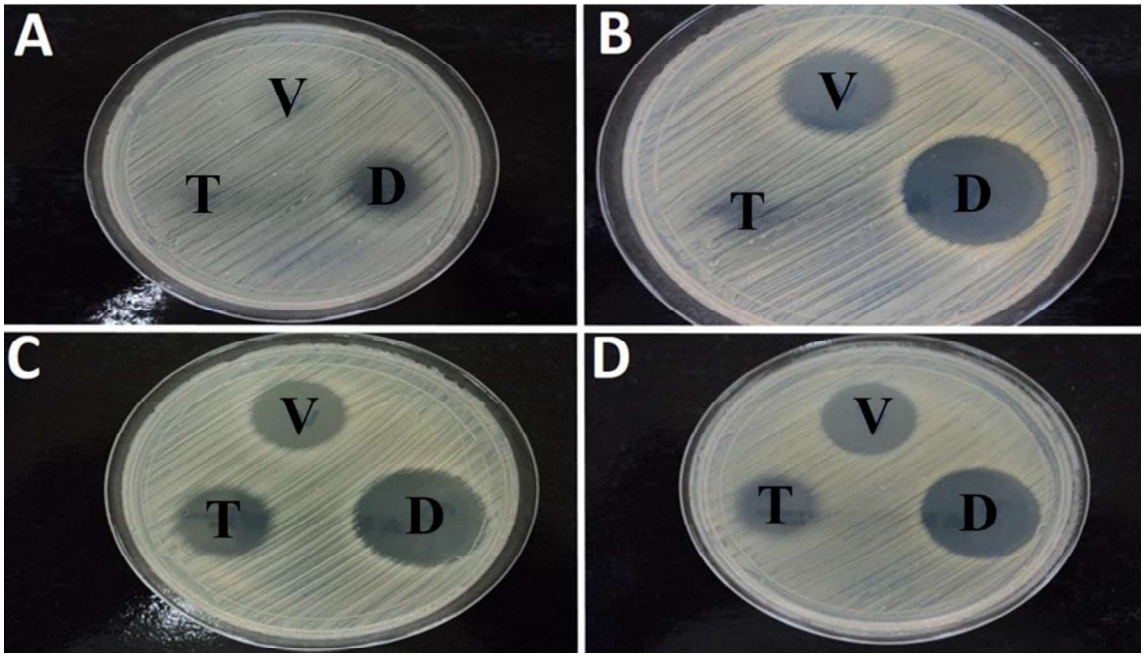

Figure 4 - The pictures above illustrate prediffusion described above, highlighting the control strains: (A) Mu3 ATCC 700698 (hVISA), (B) MU50 ATCC 700699 (VISA) and two clinical isolates, (C) SI11 and(D) L10, both having the hVISA phenotype. V – vancomycin; T – teicoplanin; D – daptomycin.

DISCUSSION

The prediffusion method is an alternative to conventional methods, allowing antimicrobials with high molecular weight to be evaluated by this alternative disk diffusion, since by conventional methodology, they do not have cutoffs in CLSI guidelines.

Surprisingly, few studies have been published using prediffusion for the assessment of *Staphylococcus aureus* susceptibility to glycopeptides and daptomycin (Nielsen & Casals, 2005; Katz, Luperchio & Thorne, 2008). The major disadvantage of prediffusion considering glycopeptide susceptibility is that the methodology is unable to differentiate between hVISA and VISA isolates. Besides, it is a qualitative methodology without the possibility to determine MICs. Its qualitative data have presented a good correlation with MIC results, suggesting this test may be used as an alternative test.

In this study, predifusion data for daptomycin were also highly consistent with MIC results, which were also observed by other authors (Katz, Luperchio & Thorne, 2008). Altogether, these data may support the clinical use of predifusion test. However, more data must be generated to confirm this hypothesis

Since 2009, CLSI no longer recommends disk diffusion methodology for vancomycin, requiring clinical laboratories to determine the MIC. Despite being a microdilution reference test, MIC testing is somehow laborious and requires validation. Therefore, several laboratories use the Etest® as an alternative methodology to determine MIC, which is more expensive and tend to overestimate the MIC (Van Hal et al., 2011). However, it is practical and shows a strong correlation with clinical results, justifying its wide usage.

A meta-analysis published in 2012 correlated vancomycin MIC values determined by Etest® with therapeutic failure. The study found a significant correlation

between isolates having a MIC ≥ 1.5 µg/mL and therapeutic failure, with an odds ratio of 1.74 (95% CI: 1.34 to 2.21; p < 0:01) (Van Hal, Lodise & Paterson, 2012). Thus, we used the MICs determined by Etest, in an attempt to find a correlation between the results of pre-diffusion and Etest.

Several phenotypic tests have been used to detect hVISA as the hetero-resistant phenotype has no reliable molecular characterization marker(s) and required the use of phenotypic tests with varying sensitivities and specificities. Satola and colleagues reported that screening on BHI agar with 4 µg/ml of vancomycin presents 91% sensitivity and 94% specificity rates, while the Etest® macromethod shows a sensitivity of 57 % and specificity of 96 % and Etest ® GRD shows a sensitivity of 57 % and specificity of 97 % (Satola et al., 2011).

This study has some limitations. First, the methodology used to determine the MIC was the Etest®, even though the gold standard is the broth microdilution. Second, all isolates tested were susceptible to daptomycin, making it necessary to evaluate the performance of the prediffusion resistant isolates to determine if the method may present good accuracy for this antimicrobial agent.

These data indicated that when comparing the prediffusion method used for detecting hVISA, the specificity (83.1 %) and sensitivity (91.7 %) rates from this study were similar to other widely used tests. The results demonstrated that, despite being infrequently used, this test could be viable and effective in screening clinical isolates for a hVISA phenotype. The high negative predictive value (97.1 %) allows for testing negative, exclude the possibility of that the phenotype. Taking into account that all positive screening tests should be confirmed with PAP-AUC, the prediffusion method, as a simple and low cost test, should be considered not only as a useful test to assess the

susceptibility of *S. aureus* to glycopeptides and daptomycin, but also as a screening test for hVISA.

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V. ARTIGO 2.

Artigo submetido ao periódico: *Canadian Journal of Infectious Diseases & Medical Microbiology*, em 23 de setembro de 2014. Fator de Impacto: 2,225

Evaluation of the Accuracy of Phenotypic Methods for the Detection of Heteroresistant Vancomycin-Intermediate *Staphylococcus aureus* (hVISA)

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Background: The detection of heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hVISA) is challenging because these strains are susceptible to vancomycin in vitro and are therefore categorized as susceptible by the usual laboratory methods. **Objective:** The objective of this study was to analyze the performance of the main phenotypic tests available to characterize heteroresistance to vancomycin. **Methods:** A total of 124 methicillin-resistant *Staphylococcus aureus* (MRSA) were isolated from hospitals in the state of Santa Catarina, Brazil. Etest glycopeptide resistance detection (GRD) was performed according to the manufacturer's instructions. The Etest macromethod was performed using a 2.0 McFarland inoculum on brain-heart infusion (BHI) agar plates, using vancomycin Etest strips. Four 10 µL droplets from 0.5 McFarland suspension were dropped by a pipette onto the BHI with casein and 4 µg/mL vancomycin agar screening plates. Population analysis profile-area under the curve (PAP-AUC) was performed as described by Wootton et al. **Results:** The GRD methodology had a sensitivity of 66.7% and specificity of 97.3%. The Etest macromethod sensitivity was 75% with a specificity of 94.6%. The screening with BHI agar with casein and 4 µg/mL of vancomycin showed a sensitivity of 90.9% and specificity of 93.8%. The GRD presented the greatest accuracy (94.3 %), but was very similar to the other two tests. **Discussion:** Due to its high sensitivity and high negative predictive value, the agar screening method is an excellent screening test because truly negative samples can be identified. The agar screening method is a simple, inexpensive

and easily employable method in routine diagnostic laboratories, including small laboratories. **Conclusion:** We conclude that the detection of hVISA, although challenging, is essential to the selection of the correct antibiotic therapy and the replacement of vancomycin with some other drug, such as linezolid or daptomycin. The methods used routinely to detect vancomycin resistance vary in sensitivity and specificity, and may fail to detect hVISA.

Journal Keywords: MRSA; Glycopeptides; Antimicrobial resistance

INTRODUCTION

Vancomycin-intermediate *Staphylococcus aureus* (VISA), a phenotype characterized by cell wall thickness, is becoming increasingly common during prolonged therapy with glycopeptides[1].

Colonies of heteroresistant vancomycin-intermediate *S. aureus* (hVISA) are heterogeneous in appearance and pigmentation, giving the impression of contamination and potentially confusing microbiologists[2]. The mechanism of hVISA resistance is associated with the activation of cell wall synthesis, which increases the production of waste mucopeptide and reduces the amount of antibiotic that reaches the site of action, resulting in cell wall thickening and subsequent sequestration of the drug[3]. It has been speculated that hVISA may be a precursor of VISA; after prolonged exposure to antimicrobials, the selection of a homogenous population of hVISA cells may occur, leading to the expression of a VISA phenotype[4].

The detection of hVISA is challenging because these strains are susceptible to vancomycin *in vitro* (minimum inhibitory concentration (MIC) $\leq$ 2 μg/mL) and are therefore categorized as susceptible by the usual laboratory methods. However, the presence of a subpopulation representing 1 in 10^6 bacterial cells that can grow in the

presence of 4 μg/mL vancomycin may lead to treatment failure in patients treated with vancomycin[5,6].

A meta-analysis published in 2011 revealed that the rates of treatment failure (designated as persistent infection or bacteremia) related to hVISA were 2-fold greater than to infections caused by vancomycin-susceptible *S. aureus* (OR: 2.37, 95 % CI: 1.53-3.67)[6]. Therefore, the development of accurate and practical methods for the detection of hVISA is of increasing importance[7].

Reference methods used to evaluate susceptibility, such as broth microdilution, fail to detect hVISA, partly due to the small size of the inoculum, the relatively poor growth of hVISA on Mueller-Hinton agar, and the short incubation period of only 24 hours. The inoculum size is critical for the detection of subpopulations of resistant cells. In addition, hVISA strains are characteristically slow growing, with thicker cell walls and unique pleomorphic characteristics, and produce colonies of varying sizes and nutritional requirements[8].

Other screening methods, such as the macro Etest, Etest glycopeptide resistance detection (GRD) and agar screening, use enriched media, a denser bacterial inoculum (2 McFarland scale) and prolonged incubation (48 hours) but have variable sensitivity and specificity; thus, it is difficult to obtain an accurate diagnosis using a single test. Because hVISA is a heterogeneous, minority subpopulation, there are no reliable molecular markers to detect heteroresistance[9].

Population analysis profile-area under the curve (PAP-AUC) has been the most reliable and reproducible approach and is considered to be the gold standard for hVISA confirmation. PAP-AUC was specifically designed for discriminating hVISA and VISA. This method analyzes the presence of different subpopulations using serial concentrations of vancomycin to quantify the viable bacterial populations at each

vancomycin concentration. PAP-AUC is a very laborious and expensive method and is inappropriate for routine use in clinical laboratories[10].

Knowledge of local epidemiological data and early detection of hVISA may assist in the selection of appropriate antimicrobial agent for treatment, decreasing the morbidity and mortality associated with infection caused by hVISA[11].

The objective of this study was to analyze the performance of the main phenotypic tests available to characterize heteroresistance to vancomycin.

METHODS

Bacterial samples

We used 124 clinical isolates of methicillin-resistant *S. aureus* (MRSA) obtained from various anatomical sites from patients in three hospitals in Florianópolis (Hospital Caridade, Hospital Governador Celso Ramos and SOS Cardio) and a hospital in Blumenau (Hospital Santa Isabel), all located in the state of Santa Catarina in southern Brazil. Samples were collected from February 2009 to February 2013. We discarded isolates from the same patient. All isolates were used and there was no selection bias. The biochemical identification was confirmed by Gram, catalase, mannitol, coagulase and Dnase.

MIC determination

Vancomicin and teicoplanin MICs were determined by Etest® (BioMérieux, Marcy l'Etoile, France), following the manufacturer's instruction and CLSI interpretative criteria[12]. To ensure the quality and accuracy of the test results,

Staphylococcus aureus strains ATCC 29213 (MSSA), ATCC 43300 (MRSA), ATCC 700698 (hVISA) and ATCC 700699 (VISA) were used.

Agar screening

To verify the ability of the isolates to grow in the presence of vancomycin, BHI agar plates containing 4 µg/mL vancomycin and 16 g/L pancreatic digest of casein were used. A 10-µL aliquot of a 2.0 McFarland was inoculated on the plates and incubated at 35°C for 48 hours. The growth of 2 or more colonies was considered to be a positive test for hVISA[13].

Etest GRD

The Etest GRD® method (BioMérieux, Marcy l'Etoile, France) utilizes different concentration gradients (0.5 to 32 µg/mL) of vancomycin and teicoplanin. The inoculum was adjusted to the 0.5 McFarland standard and inoculated on Mueller-Hinton agar containing 5% sheep blood, according to the manufacturer's recommendations. Readings were obtained at 24 and 48 hours and considered to be positive for hVISA isolates with an MIC of 8 µg/mL for teicoplanin and vancomycin[14].

Etest macromethod

A high bacterial inoculum (2 McFarland scale) was used to inoculate nutritionally enriched medium (BHI), followed by prolonged incubation (48 hours). A 200-µL aliquot of the bacterial suspension was seeded onto a BHI agar plate. Etest® (BioMerieux, Marcy l'Etoile, France) vancomycin strips were added, and the plates were incubated at 35°C for 48 hours. A test was considered to be positive for hVISA

when the MIC for teicoplanin was 12 μg/mL or when an MIC of 8 μg/mL for teicoplanin and vancomycin was obtained[14].

PAP-AUC

After incubation on solid medium, the bacteria were diluted in sterile saline at dilutions ranging from 10^{-1} to 10^{-8} and subsequently spotted as 10-μL spots on BHI agar plates containing 0, 0.5, 1, 2, 3, 4, 5, 6 and 8 μg/mL of vancomycin, respectively. The plates were incubated for 48 hours, and the colonies were counted to determine the $\log_{10}$CFU/mL; these data were then plotted on a graph as a function of the vancomycin concentration. The AUC was calculated using the strain Mu3 (ATCC 700698) as a control. To confirm the designation as hVISA, the ratio of the AUC of the isolate to that of the Mu3 strain was required to be greater or equal to 0.9 and non-hVISA isolates had a PAP-AUC < 0.9[10,13].

RESULTS

For the antimicrobials vancomycin and teicoplanin, all 124 isolates were susceptible (Figure 1). All vancomycin MICs were equal to or greater than 0.5 μg/mL, reaching 3 μg/mL for seven isolates. To confirm these 7 isolates, the MICs were determined by the broth microdilution. Thus, three showed a MIC of 1 μg/mL and 4 with a MIC of 2 μg/mL. Therefore, all were considered to be susceptible to vancomycin (MIC $\leq$ 2 μg/mL). The MICs of teicoplanin were much higher than those of vancomycin, with MICs up to 12 μg/mL. The isolate with MIC 12 μg/mL for teicoplanin was regarded as sensitive as the MIC obtained by broth microdilution was 4 μg/mL (MIC $\leq$ 8 μg/mL).

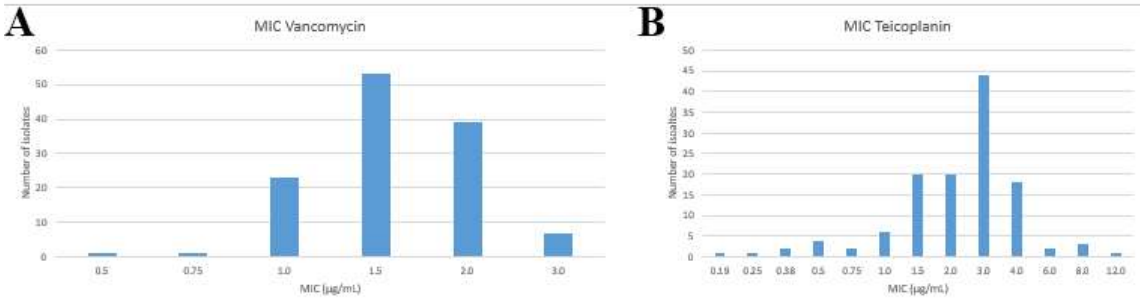

Figure 1 – MIC to vancomycin and teicoplanin determined by Etest. In A, the results for vancomycin are shown. Seven isolates would be considered VISA by the Etest methodology, but their MICs determined by the reference methodology were ≤ 2 µg/mL. In B, susceptibility to teicoplanin is shown, with only one isolate presenting an MIC > 8 µg/mL, which was not confirmed by broth microdilution.

Screening tests for the detection of hVISA isolates were performed. The results for the isolates that were confirmed as hVISA are listed in Table 1.

Table 1 - Results of screening tests and PAP-AUC for the confirmation of hVISA (PAP-AUC values higher than 0.9 confirm this phenotype).

Isolate number	screening[a]/ macromethod[b]/ GRD[c]	AUC ratio[d]
SI4	negative/positive/negative	1.14
SI11	positive/positive/positive	0.99
SI13	positive/negative/positive	1.19
L10	positive/negative/positive	0.92
L36	positive/negative/negative	1.02
L43	positive/positive/negative	0.98
L54	positive/positive/positive	1.08
L69	positive/negative/positive	0.93
L74	positive/positive/positive	1.17
L80	negative/positive/positive	1.12
L84	positive/negative/positive	1.11
L92	negative/positive/postive	0.99

[a] – agar screening in brain-heart infusion (BHI) with 4 µg/mL vancomycin and 16 g/L pancreatic digest of casein
[b] – Etest macromethod
[c] – Etest glycopeptide resistance detection®
[d] – ratio of isolate AUC/Mu3 AUC

Based on the above results, the sensitivity, specificity, positive and negative predictive values, and accuracy of each test were calculated (Table 2). No test yielded optimum values for all variables; the agar screening had the best sensitivity (90.9 %) and negative predictive value (99.1 %), while the GRD had higher specificity (97.3 %), positive predictive value (72.3 %) and accuracy (94.3 %). Although the Etest macromethod was not superior to the other tests, its accuracy was very similar (92.7 %).

Table 2 - Parameters of the main screening tests for the detection of hVISA.

Methodology	Sensitivity	Specificity	PPV[a]	NPV[b]	Accuracy
Etest GRD[c]	66.7%	97.3%	72.3%	96.5%	94.3%
Etest macromethod	75%	94.6%	60%	97.2%	92.7%
Agar screening[d]	90.9%	93.8%	58.8%	99.1%	93.5%

[a] – positive predictive value
[b] – negative predictive value
[c] – Etest glycopeptide resistance detection®
[d] – agar screening in brain-heart infusion (BHI) with 4 µg/mL vancomycin and 16 g/L pancreatic digest of casein

DISCUSSION

Because heteroresistance is usually associated with previous vancomycin use, *S. aureus* with elevated MICs (≥ 2 µg/mL) is among the main risk factors for the development of this phenotype. The selection of an appropriate treatment strategy depends on the methodology because the Etest has a tendency to overestimate MICs[15]. In this study, the hVISA isolates had an MIC ≥ 1.5 µg/mL (Etest). Some studies[16,17] have demonstrated a relationship between an MIC ≥ 1.5 µg/mL and the development of hVISA, using the Etest. Other studies using the same methodology[18,19] have presented data demonstrating an association of an MIC < 1.0 µg/mL with hVISA. Thus, a higher

206 MIC (< 4.0 µg/mL) is associated with an increased likelihood of the hVISA phenotype.
207 Isolates with an MIC > 4.0 µg/mL are considered to be VISA.

208 Because the characteristics of hVISA are heterogeneous and constitute a
209 minority of the bacterial population, there are no recommended methods for molecular
210 detection. Phenotypic methods with large bacterial inoculums, enriched culture media
211 and prolonged incubation times are needed[8]. These requirements can be fulfilled
212 through the use of the three methods utilized in this study in combination.

213 The Etest GRD uses rich medium (sheep blood) and a prolonged incubation time
214 but a traditional inoculum (0.5 McFarland scale). Cost is a disadvantage of the Etest
215 GRD, but its ease of standardization justifies its use. It has a sensitivity of 57-93% and a
216 specificity of 82-97%, depending on the study[13,14,20]. We observed a sensitivity of
217 66.7% and a specificity of 97.3%, in agreement with international studies with larger
218 samples.

219 The Etest macromethod combines the three key features for the detection of
220 hVISA: large inoculum, prolonged incubation time and nutrient medium (BHI). It is a
221 fast and simple method that utilizes strips of vancomycin and/or teicoplanin to evaluate
222 glycopeptide susceptibility. This method has a sensitivity of 57-89 % and a specificity
223 of 55-96 % study[13,14,20]. Our data indicated a sensitivity of 75 % and a specificity of
224 94.6 %.

225 Finally, agar screening in BHI with 4 µg/ml vancomycin and 16 g/L pancreatic
226 digest of casein can be used with an inoculum of 0.5 or 2 McFarland standard and an
227 incubation time of 48 hours in enriched medium. This test yields the best sensitivity and
228 specificity, 91 % and 94 %, respectively[13], similar to the results obtained in our study
229 (90.9 % sensitivity and 93.8 % specificity). These characteristics permit the use of this
230 method for hVISA screening in association with (PAP-AUC) confirmatory testing. A

disadvantage of this method is the need for standardization; these standards must be prepared in house, and thus the concentration of vancomycin can vary, thus negatively affecting test performance.

Because the performance of these screening tests is inconsistent, some authors[8,14] have recommended using these tests in combination to improve sensitivity—a strategy that may increase costs[14]. Alternatively, a test with high sensitivity could be combined with a test with high specificity.

Based on the results of this study and those of studies with larger population sizes, we suggest a flowchart for screening and confirming hVISA isolates (Figure 2). Due to its high sensitivity and high negative predictive value, the agar screening method is an excellent screening test because truly negative samples can be identified. The agar screening method is a simple, inexpensive and easily employable method in routine diagnostic laboratories, including small laboratories. How the entire suspected hVISA should be confirmed by PAP-AUC, the screening agar can be an excellent alternative.

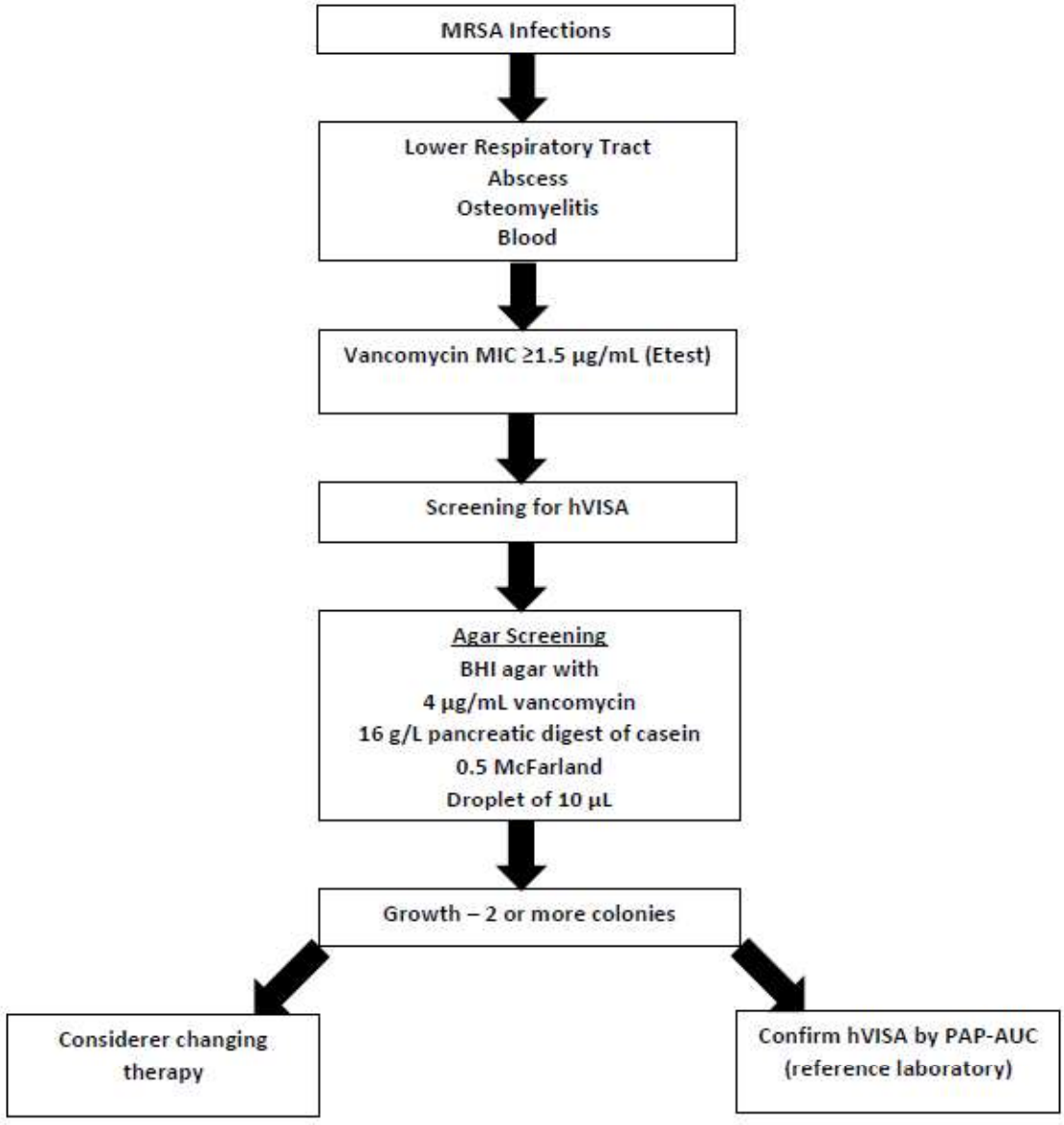

Figure 2 - Flowchart for hVISA screening. Assuming that hVISA is more common in chronic infections and/or anatomical regions with high bacterial inocula, it is likely that bone and lung infections are a major cause of hVISA infections. Because bloodstream infections are those with the highest mortality rates, the criteria of anatomical site and vancomycin MIC can be used to indicate the need for hVISA screening tests. Isolates with an MIC < 1.5 µg/mL can be excluded because they are associated with a lower prevalence of hVISA. The agar screening test should be used, and positive results should be reported to the doctor immediately to permit a change in therapy. Because performing PAP-AUC as a confirmatory test is impractical for routine laboratories, suspected hVISA isolates should be referred to a reference laboratory (adapted from[8,14,21]).

We conclude that the detection of hVISA, although challenging, is essential to the selection of the correct antibiotic therapy and the replacement of vancomycin with some other drug, such as linezolid or daptomycin. The methods used routinely to detect

vancomycin resistance vary in sensitivity and specificity, and may fail to detect hVISA. The appropriate use of screening tests will depend on the prevalence rates of hVISA in each institution, and the use of a single screening test will yield poor results. A viable alternative would be to establish a flowchart for processing samples that includes a choice of tests that are suitable for routine epidemiology and are inexpensive. The combination of the three methods may be the best alternative because the ones with vancomycin MIC $\leq$ 2 μg/mL, may have heteroresistance and, in these cases, the correct characterization of hVISA may impact directly in the therapeutic success.

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VI. ARTIGO 3.

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Molecular Epidemiology of the First Hetero-Resistant Vancomycin Intermediate *Staphylococcus aureus* (hVISA) Described in Brazil

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ABSTRACT

To determine the epidemiological and molecular characteristics of twelve *Staphylococcus aureus* isolates presenting hetero-resistance to vancomycin (hVISA) in laboratories of two cities in Santa Catarina, southern Brazil. Epidemiological data, including the city of isolation, health institution, and date of isolation were considered, as well as the associated clinical specimen. For molecular characterization, we analyzed the staphylococcal cassette chromosome (SCC*mec*) types, the *erm* gene presence and the genomic diversity of isolates using pulsed-field gel electrophoresis (PFGE). The twelve isolates of *S. aureus* were previously confirmed as hVISA using the population analysis profile-area under curve (PAP-AUC) Regarding genetic variability, three clones were detected: the main one (clone A) composed of four isolates and two other clones (B and C), with two isolates each. For clone A, two isolates presented identical band patterns and were related to the same hospital, with an interval of fifty-seven days between their isolation. The other isolates of this clone showed no epidemiological link between them because they were isolated in different hospitals and had no temporal relationship. The other two clones showed no detectable epidemiological relationship. The hVISA recovered in Santa Catarina State from 2009 to 2012 had, in general, heterogeneous genomic patterns based on PFGE results, which is in accordance with the fact that these isolates had little or no epidemiological relationship among them. Due to the characteristic instability phenotype and often prolonged vancomycin therapy for selection, clonal spread is not as common as for other resistance mechanisms disseminated through horizontal gene transfer.

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INTRODUCTION

Staphylococcus aureus can acquire, during prolonged therapy with glycopeptides, a phenotype of resistance very peculiar. Through selective pressure and apparently without transfer of genetic material, may suffer mutations in genes responsible for production of cell wall, making it thicker and less susceptible to the action of antimicrobials (Howden et al., 2008).

The hetero-resistance of *S. aureus* to vancomycin (hVISA) causes changes in the macro-morphological features of the colonies, who present with heterogeneous appearance and pigmentation, giving the impression of contamination and may confuse the microbiologist (Severin et al., 2004). Its mechanism of resistance is associated with activation of cell wall synthesis, which increases the production of waste mucopeptide and reduces the amount of antibiotic that reaches the site of action, thus causing cell wall thickening and subsequent imprisonment drug (Rybak et al., 2008). Speculated that hVISA could be regarded as precursors of VISA strains, once, after prolonged exposure to antimicrobial, selection homogenous population of cells may occur, expressing the phenotype VISA. This phenotype, plus minority, is unstable (Longzhu et al., 2006).

The detection of infections caused by hVISA represents a challenge for microbiologist, since these strains are considered susceptible to vancomycin *in vitro* (minimum inhibitory concentration (MIC) $\leq$ 2 µg/mL), and therefore categorized as susceptible by the usual laboratory methods (Howden et al., 2010). However contains subpopulations 1 in each 10^6 bacterial cells, that can grow in the presence of 4 µg/mL of vancomycin, may lead the patient to therapy with vancomycin with possible treatment failure (Satola et al., 2011a; Van Hal & Paterson, 2011).

Reference methods used to evaluate the susceptibility as broth microdilution, Etest and automated methods fail to detect hVISA. Because the phenotype is a heterogeneous phenomenon, reliable molecular markers to confirm this phenotype have

not yet been found. Partly due to the inoculum be small, relatively poor growth on Mueller-Hinton agar or incubation for only 24 hours. The inoculum size is critical to the detection of subpopulation of resistant cells, furthermore hVISA strains are notoriously slow growing, with cell walls thicker and pleomorphic unique characteristics, with colonies of varying sizes and nutritionally exacting (Satola et al., 2011b).

The population analysis profile-area under the curve (PAP-AUC) has been the most reliable and reproducible approach, being considered the gold standard test for hVISA (Wootton et al., 2001). Was specifically designed for discriminating hVISA and VISA. It is a method of analysis of modified sub-populations using serial concentrations of vancomycin, in order to quantify the viable bacterial populations in such concentrations. It is a very laborious, expensive and inappropriate for routine use in clinical laboratories (Yusof et al., 2008).

A meta-analysis published in 2011, the rates of treatment failure (designated as persistent infection or bacteremia) related to hVISA isolates were 2 times more common than in infections caused by *S. aureus* susceptible to vancomycin (OR: 2.37, 95% CI: 1.53-3.67) (Van Hal & Paterson, 2011). Therefore, an accurate and practical for the detection of hVISA are isolated from clinical laboratory method is of increasing importance (Leonard et al., 2009).

Despite the controversy between studies regarding the association of hVISA and mortality, knowledge of the epidemiological profile is very important in assisting the clinician when choosing the appropriate antibacterial therapy. The objective of this study is to evaluate the phenotypic and molecular epidemiology characteristics of hVISA isolates in the state of Santa Catarina, Brazil.

METHODS

Bacterial samples

We used 12 clinical isolates of hVISA obtained from various anatomical sites from patients in three hospitals in Florianópolis (Hospital Caridade, Hospital Governador Celso Ramos, and SOS Cardio) and a hospital in Blumenau (Hospital Santa Isabel), all located in the state of Santa Catarina in southern Brazil. Samples were collected from November 2009 to October 2012.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method, according to the recommendations and interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI, 2013). We also performed the D test for the detection of inducible resistance to clindamycin. Disks of erythromycin and clindamycin were placed 26 mm apart, and a flattening of the inhibition zone indicated a positive test, which was reported as clindamycin resistance. Vancomycin MICs were determined by macrodilution method (CLSI, 2013).

PAP-AUC

After incubation on solid medium, the bacteria were diluted in sterile saline at dilutions ranging from 10-1 to 10-8 and subsequently spotted as 10-µL spots on BHI agar plates containing 0, 0.5, 1, 2, 3, 4, 5, 6 and 8 µg/mL of vancomycin. The plates were incubated for 48 hours, and the colonies were counted to determine the log10CFU/mL; these data were then plotted on a graph as a function of the vancomycin concentration. The AUC was calculated using the strain Mu3 (ATCC 700698) as a control. To confirm the designation as hVISA, the ratio of the AUC of the isolate to that

of the Mu3 strain must be greater or equal to 0.9 and non-hVISA isolates had a PAP-AUC < 0.9 (Wootton et al., 2001; Satola et al., 2011b).

Multiplex PCR for the detection of the staphylococcal cassette chromosome (SCC*mec*)

The SCC*mec* type was determined using the multiplex PCR method according to the protocol developed by Zhang et al. The amplicons that were formed had the following sizes: I (613 bp), II (398 bp), III (280 bp), IVa (776 bp), IVb (493 bp), IVc (200 bp), IVd (881 bp), and V (325 bp) (Zhang et al., 2003; Oliveira & Lencastre, 2002).

PCR for *erm* gene detection

For isolates with positive results in the phenotypic test for inducible resistance to clindamycin, *erm* gene PCR amplification was performed according to the multiplex PCR protocol developed by Khan et al (1999). The PCR product (610 bp for *erm*A and 520 bp for *erm*C) was analyzed by electrophoresis through a 1.5% agarose gel (Khan et al., 1999; Lina et al., 1999).

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to McDougal et al (2003) and Pinto et al (2013). The fragments were subjected to PFGE using 1% agarose gels (Pulsed Field Certified Agarose; Bio-Rad) in 0.5X Tris-borate-EDTA buffer with a CHEF-DR III system (Bio-Rad). The gels were stained with 0.5 µg/mL ethidium bromide, visualized under UV light, and photographed using a GelDoc™ XR System (Bio Rad). The PFGE patterns were analyzed using Bionumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). The PFGE patterns were clustered by UPGMA. A dendrogram was generated

from a similarity matrix calculated using the Dice similarity coefficient with an optimization of 0.5% and a tolerance of 1%. PFGE clusters were defined as isolates with a similarity of 80% or higher on the dendrogram (Tenover, 1995).

RESULTS

The twelve hVISA isolates were isolated from tracheal aspirates (n=5), osteomyelitis (n=4), blood (n=1), a skin lesion (n=1) and a surgical wound (n=1).The resistance of the 12 hVISA isolates to antimicrobial agents was assessed by disk diffusion, with the following results: clindamycin (92.7%), erythromycin (100%), trimethoprim/sulfamethoxazole (16.7%), ciprofloxacin (92.7%), tetracycline (16.7%), chloramphenicol (8.3%) and gentamicin (33.3%). All isolates were considered susceptible to linezolid and teicoplanin. The vancomycin MICs were 1.0 µg/mL (33.3 %) and 2.0 µg/mL (66.7 %).

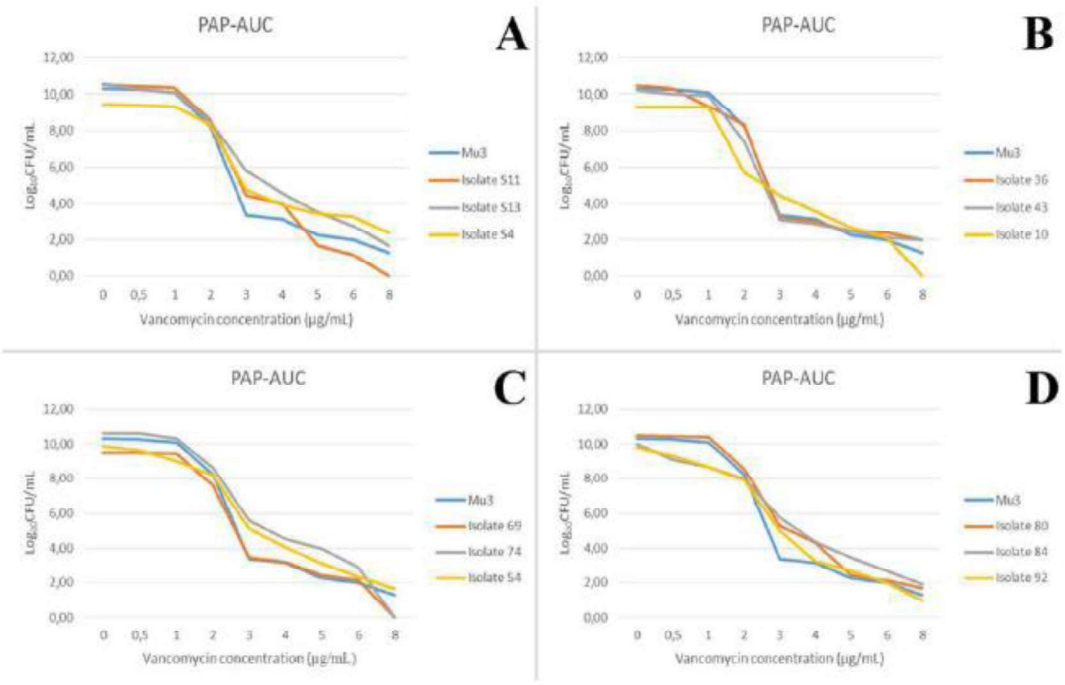

Figure 1 - Growth curves of the twelve isolates characterized as hVISA compared to the standard strain Mu3. All isolates showed an AUC/Mu3 AUC ratio greater than 0.9. For

clarity, bacterial growth was expressed in log_{10}CFU/mL. Nine concentrations of vancomycin (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 8 μg/mL) were used. Graph A depicts isolates S4, S11 and S13. Graph B shows isolates L10, L43 and L36. Graph C shows isolates L54, L69 and L74. Graph D shows isolates L80, L84 and L92. The reference strain Mu3 is shown in all graphs.

Only two isolates (10 and 54) exhibited inducible clindamycin resistance, as determined by a positive D test. These isolates were subjected to PCR for *erm* gene detection, and both contained the *erm*A gene. Isolate 84, which was resistant to erythromycin and sensitive to clindamycin, yielded a negative D test, and the *erm* gene was not amplified. All other isolates showed constitutive clindamycin resistance.

Isolate SI4 contained two SCC*mec* types (I and II). Isolate 84 contained SCC*mec* type I. Isolates S11, 36, 54, 69, 80 and 92 contained SCC*mec* type II. SCC*mec* type III was observed in isolates SI13, 10 and 43. Although isolate 80 was considered to be a community isolate, it did not contain SCC*mec* type IV, which is frequently associated with community-acquired methicillin-resistant *S. aureus* (CA-MRSA). Only one isolate showed SCC*mec* type IV (IVc), 74.The isolation of the microorganism in the first 48 hours of admission was used as the criterion for classification as CA-MRSA.

PFGE was used to assess the degree of genetic similarity, and the results are presented in Figure 2. Three clones were observed: a primary clone (A), comprising four isolates (SI4, L54, L69, and L92); clone B, comprising isolates L36 and L80; and clone C, comprising isolates SI13 and L43. Isolates SI11, L10, L74, and L84 did not display genetic similarity with any of the other isolates. The criteria established by Tenover were used for classification, with a similarity index of 80%.

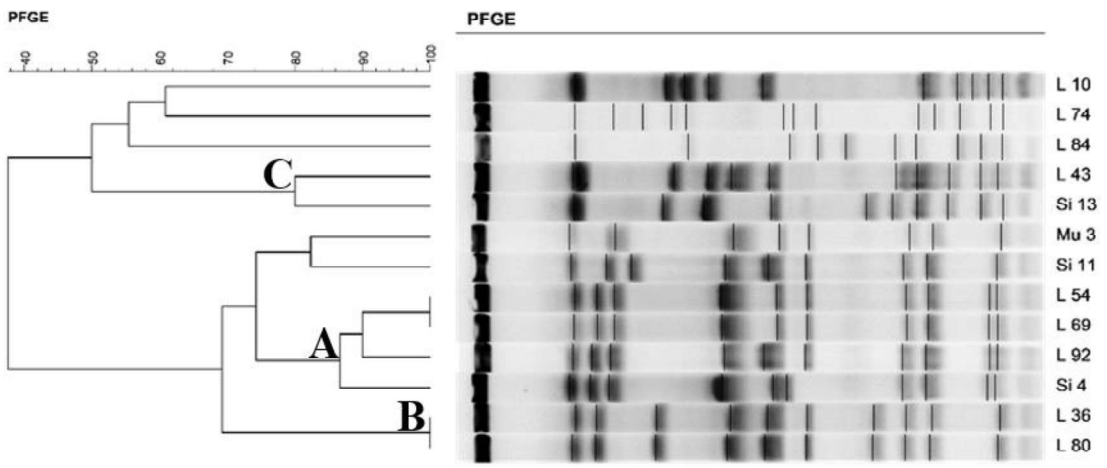

Figure 2 - Dendrogram showing the similarities between the twelve hVISA isolates. Three clones were observed: one comprising four isolates (A), two of which displayed 100% similarity (L54 and L69); clone B, which comprised two isolates that were genetically indistinguishable (L36 and L80); and clone C, which comprised two isolates (SI13 and L43). The SI11, L10, and L74 isolates did not exhibit any similarity with the other isolates.

In addition to the molecular characteristics described above, Table 1 presents the city of isolation, attended institution, biological sample, and isolation data. The samples were isolated in two different cities that are 120 kilometers apart. In the city of Florianópolis, the state capital of Santa Catarina, three hospitals and two clinics were included. Approximately 33 months separated the first and last isolates. Isolates L54 and L69 were isolated in the same hospital, have 100% genetic similarity, and were isolated from patients with osteomyelitis approximately 57 days apart, which might indicate a common route of infection.

Table 1 - Epidemiological and clinical characteristics of the twelve hVISA isolates.

Isolate number	City	Institution	Clinical sample	SCC*mec*	Clone	Date
10	Florianópolis	Hospital A	Osteomyelitis	III	Non clonal	23/11/2009
36	Florianópolis	Hospital A	Tracheal aspirate	II	B	03/09/2010
43	Florianópolis	Hospital A	Surgical wound	III	C	14/10/2010
54	Florianópolis	Hospital A	Osteomyelitis	II	A	04/05/2011
69	Florianópolis	Hospital A	Osteomyelitis	II	A	01/07/2011
74	Florianópolis	Hospital B	Skin lesion	IVc	Non clonal	22/12/2011
80	Florianópolis	Clinic A	Tracheal aspirate	II	B	09/01/2012
84	Florianópolis	Hospital A	Tracheal aspirate	I	Non clonal	25/02/2012
92	Florianópolis	Hospital C	Tracheal aspirate	II	A	18/08/2012
SI4	Blumenau	Hospital D	Blood	I,II	A	28/09/2010
SI11	Blumenau	Hospital D	Tracheal aspirate	II	Non clonal	20/02/2011
SI13	Blumenau	Hospital D	Osteomyelitis	III	C	12/04/2011

DISCUSSION

As far as we know, this is the first description of hVISA in Brazil. Two brazilian studies reported decreased susceptibility to vancomycin, but not confirm hVISA phenotype. In 2001, Oliveira and colleagues reported 5 clinical isolates of MRSA with MIC of 8 µg/mL, which was, based on available criteria (NCCLS-1997), named VRSA. However, isolates did not harboured van genes and, as well demonstrated by authors, presented a cell wall thickening (Oliveira et al., 2001). In 2006, Lutz and Barth described 18 clinical isolates as possible hVISA, since they were positive for screening test (BHI with 4 µg/mL of vancomycin). However, confirmatory test for hVISA, the PAP-AUC was not performed, especially because, on that time, the nowadays accepted criteria for confirmation of this phenotype were not broadcast (Lutz & Barth, 2006; Lutz et al., 2003). Based on these data, we believe our study provides the first report of isolation of hVISA in Brazil.

The anatomical sites most frequently associated with hVISA infections are those with a higher bacterial inoculum (abscesses, pneumonia) and those associated with chronic infections (endocarditis, osteomyelitis) for which the use of vancomycin for

prolonged periods is quite common and the low penetration of the antibiotic in these sites favors the development of resistance (Satola et al., 2011b). Several studies (Rybak et al., 2008; Park et al., 2012; Hu et al., 2013; Richter et al., 2011; Horne et al., 2009) have established that the blood, lower respiratory tract, skin wounds, abscesses and osteomyelitis are the most common sites of isolation of hVISA. These data are similar to those of the present study.

The isolates exhibited a profile of heterogeneous susceptibility, characterized by low resistance to chloramphenicol and trimethoprim/sulfamethoxazole, drugs that are used infrequently to treat MRSA infections. Other studies (Park et al., 2012; Richter et al., 2011) have reported low rates of resistance to sulfonamides in hVISA isolates, ranging from 9 to 9.9 %. By contrast, studies of samples from Korea (Gyungtae et al., 2010) and China (Hu et al., 2013) revealed much higher values, 58 and 54 %, respectively. A chloramphenicol resistance rate of 16.7 % was observed in our study, while a rate of 47.6 % was observed in a study by Hu and colleagues in 2013. The rates of resistance to macrolides, lincosamides, aminoglycosides, fluoroquinolones and tetracyclines are high, with values greater than 64 %, precluding its empirical use (Park et al., 2012; Hu et al., 2013; Richter et al., 2011; Gyungtae et al., 2010) . Knowledge of the local susceptibility profile is essential for the maintenance of adequate empirical therapy.

Clindamycin is a therapeutic option for the treatment of serious infections caused by *S. aureus*, including MRSA, particularly for isolates with SCC*mec* type IV, which usually are resistant only to beta-lactams (Chua et al., 2011). However, one common mechanism of resistance is the macrolide-lincosamide-streptogramin B (MLS_B) phenotype, which expresses inducible clindamycin resistance, which cannot be detected by traditional phenotypic tests and requires the D test (Fiebelkorn et al., 2003).

In our study, two isolates exhibited inducible clindamycin resistance, and both possessed the *erm*A gene. Both were reported to be resistant to clindamycin, and thus inappropriate treatment was avoided.

There was a predominance of SCC*mec* type II, which is associated with hVISA; in MRSA, this type is associated with higher mortality rates (Hu et al., 2013). Other studies have also demonstrated a predominance of SCC*mec* type II among hVISA isolates (Hu et al., 2013; Casapao et al., 2013; Park et al., 2012; Khatib et al., 2011). Some studies have demonstrated a predominance of other types of SCC*mec* between hVISA isolates such as type I (Maor et al., 2009) and type III (Bae et al., 2009; Lin et al., 2012; Wang et al., 2013). It is interesting to note that among the 12 isolates, only 1 had SCC*mec* type IV (IVc). Although some studies did not find type IV SCC*mec* among their MRSA isolates (Maor et al., 2009; Wang et al., 2013), some other demonstrate rates as high as 16,8 % (Park et al., 2012) to 26.3% (Bae et al., 2009).

From an epidemiological perspective, it is difficult to perform further analyses due to the geographical distance and the length of time between the isolation of the first and last isolates. The microorganisms were isolated over a period of three years in different cities and even in several different institutions in the city of Florianópolis. Except for isolates L54 and L69, which are the same clone and were isolated in the same institution, the hVISA isolates did not have any characteristics that would enable an epidemiological link to be made between them.

The hVISA recovered in Santa Catarina State from 2009 to 2012 had, in general, heterogeneous genomic patterns according to the PFGE results, which is in accordance with the fact that these isolates had little or no epidemiological relationship among them.

It should be noted that due to its continental dimensions and tourist vocation, Brazil presents a great diversity of resistance mechanisms, and *S. aureus* with resistance to vancomycin (VRSA) was first described in 2014 in South America (Rossi et al., 2014). This fact demonstrates the real need for the surveillance of bacterial resistance in our country.

Epidemiological information from each health facility, as well as each geographical region, is critical to the installation of appropriate empirical therapy, especially with regard to hVISA isolates. Such bacteria have phenotypic characteristics that are difficult to correctly identify by conventional methods (MIC determination and molecular tests) and are associated with worse clinical outcomes. Due to the characteristic instability phenotype and often prolonged vancomycin therapy for selection, clonal spread is not as common as for other resistance mechanisms disseminated through horizontal gene transfer.

It is imperative that clinical microbiology laboratories detect the hVISA phenotype and associate it with the epidemiological characteristics of the patients (e.g., age, date of isolation, anatomical site, prior use of vancomycin) to provide important information for research laboratories. Through appropriate methodologies, associations between the microbiological data and the clinical characteristics of patients may enable the detection of possible associated risk factors and aid in assessing the prognosis of patients.

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453

VII. ARTIGO 4.

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Molecular Epidemiology of MRSA in Southern Brazil

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is among the most frequently isolated agents in nosocomial infections and the community. It is a constant challenge for antibacterial therapy. Therefore, it becomes essential to understand the epidemiology of MRSA isolates in the institution and/or region to guide empirical therapy. The objective of this study is to evaluate the epidemiological characteristics of MRSA isolates in the state of Santa Catarina, Brazil and determine if there is a dominant profile and clonal spread. We used 124 clinical isolates of MRSA obtained from various anatomical sites from patients in the state of Santa Catarina in southern Brazil. The profile of antimicrobial susceptibility was evaluated by disk diffusion and determination of minimum inhibitory concentration (MIC). SCC*mec* types that were present and the profile of pulsed field gel electrophoresis (PFGE) were evaluated to determine possible clones. The antimicrobial agents that have demonstrated lower rates of resistance were tetracycline (20.2%), sulfamethoxazole-trimethoprim (20.2%) and chloramphenicol (12.9%). We did not detect resistance to glycopeptides, daptomycin, linezolid and tigecycline. The predominant form was SCC*mec* type III (54%), followed by type II (21.8%). Twenty-six clonal complexes were detected without evidence of clonal spread. Despite the low prevalence of MRSA in the state of Santa Catarina, the isolates showed results that were consistent with other Brazilian studies, with a predominance of SCC*mec* type III and similar rates of antimicrobial resistance and epidemiological profile. 26 clonal complexes were detected with a wide variety of mobile genetic elements, and we noted that there was no prevalence of a given clone. As the cities of Florianopolis and Blumenau are separated by approximately 120 kilometers and the isolates were collected during a period of three years, there was no significant epidemiological relationship between the clonal complexes.

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INTRODUCTION

Staphylococcus aureus is a major agent of community-acquired infections and related health care infections. SENTRY conducted a multicenter study in Brazilian hospitals during 2005 to 2008 and found that *S. aureus* was the main agent of bloodstream infections (20.2%), the main agent of infections of the skin and soft tissue (28.1%) and the second most common agent in pneumonia in hospitalized patients (24.9%). Of these infections, approximately 30% were resistant to methicillin (MRSA)[1].

Staphylococci become resistant to methicillin by changing transpeptidases in the walls, called penicillin-binding proteins (PBPs), and lose affinity for all beta-lactam agents. This information is contained in the mecA gene responsible for encoding an altered PBP (PBP2a)[2,3].

Until the early 90s, the MRSA isolates were restricted to hospitals. Currently, however, MRSA is no longer associated with only health care infections. During this period, reports of MRSA infections acquired in the community (CA-MRSA) began in patients without assignable risk factors for acquiring MRSA (i.e., had no direct or indirect contact with the health service that could associate the MRSA infection with health care)[4].

Since then, a reverse phenomenon began to occur: the isolated community, once characterized by the presence of a staphylococcal cassette chromosome (SCC*mec*) type IV, began to be isolated in hospital settings and those types that were typically isolated in the hospital began to appear in outpatients[5,6].

With the changing pre-established profiles, it is essential to understand the epidemiology of MRSA isolates in the institution and/or region to guide empirical therapy[7]. The objective of this study is to evaluate the epidemiological characteristics of

MRSA isolates in the state of Santa Catarina and to determine if there is a dominant profile and clonal spread.

METHODS

Bacterial samples

We used 124 clinical isolates of MRSA obtained from various anatomical sites from patients in three hospitals in Florianópolis (Hospital Caridade, Hospital Governador Celso Ramos, and SOS Cardio) and a hospital in Blumenau (Hospital Santa Isabel), all located in the state of Santa Catarina in southern Brazil. Samples were collected from November 2009 to October 2012. Clinical isolates (n = 124) were collected (March 2009 to February 2013) from inpatients in three hospitals in Florianópolis (Hospital de Caridade, Hospital Governador Celso Ramos, and Cardio SOS) and a hospital in Blumenau (Hospital Santa Isabel), all located in Santa Catarina state, Southern Brazil. One isolate per patient was considered. All isolates were used, and there was no selection bias. Identification was done using the following tests: Gram staining, catalase production, mannitol fermentation, coagulase and DNase production[8].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method, according to the recommendations and interpretive criteria of the Clinical and Laboratory Standards Institute[9] and European Committee on Antimicrobial Susceptibility Testing[10]. The antimicrobials used were gentamicin (10 µg),

ciprofloxacin (5 µg), erythromycin (15 µg), clindamycin (2 µg), trimethoprim/sulfamethoxazole (1.25/23.75), chloramphenicol (30 µg), tetracycline (30 µg), teicoplanin (30 µg), tigecycline (10 µg) and linezolid (30 µg). MRSA was characterized using the cefoxitin disk (30 µg) diffusion test according to the interpretive criteria of CLSI[9], and confirmed by PCR for the gen *mec*A[11]. To detect beta-lactamase production, we used nitrocefin disk (BD BBL™ DrySlide™ Nitrocefin), using bacterial suspensions in physiological saline. *Staphylococcus aureus* ATCC 29213 was used as a positive control, and *Staphylococcus aureus* ATCC 25923 was used as a negative control.

MIC determination

Vancomycin MICs were determined by macrodilution method[9] and by Etest® (BioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions and CLSI interpretative criteria[9]. To determine the MICs of vancomycin, teicoplanin and daptomycin, Etest® was used. To ensure the quality and accuracy of the test results, *Staphylococcus aureus* strains ATCC 29213 (MSSA), ATCC 43300 (MRSA), ATCC 700698 (hVISA) and ATCC 700699 (VISA) were used.

Multiplex PCR for detecting the staphylococcal cassette chromosome (SCC*mec*)

The SCC*mec* type was determined using the multiplex PCR method according to the protocol developed by Zhang et al. The amplicons that were formed had the following sizes: I (613 bp), II (398 bp), III (280 bp), IVa (776 bp), IVb (493 bp), IVc (200 bp), IVd (881 bp), and V (325 bp)[12,13].

PCR for *erm* gene detection

For isolates with positive results in the phenotypic test for inducible resistance to clindamycin, *erm* gene PCR amplification was performed according to the multiplex PCR protocol developed by Khan et al (1999). The PCR product (610 bp for *erm*A and 520 bp for *erm*C) was analyzed by electrophoresis through a 1.5% agarose gel[14,15].

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to McDougal et al[16] and Pinto et al[17]. The fragments were subjected to PFGE using 1% agarose gels (Pulsed Field Certified Agarose; Bio-Rad) in 0.5X Tris-borate-EDTA buffer with a CHEF-DR III system (Bio-Rad). The gels were stained with 0.5 µg/mL ethidium bromide, visualized under UV light, and photographed using a GelDoc™ XR System (Bio Rad). The PFGE patterns were analyzed using Bionumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). The PFGE patterns were clustered by UPGMA. A dendrogram was generated from a similarity matrix calculated using the Dice similarity coefficient with an optimization of 0.5% and a tolerance of 1%. PFGE clusters were defined as isolates with a similarity of 80% or higher on the dendrogram[18]. For controls, we used the following SCC*mec* control strains: type I (NCTC10442); type II (N315); type III (85/2082); type IVa (CA05); type IVb (8/6-3P); type IVc (MR108); type IVd (JSC4469) and type V (WIS).

RESULTS

The prevalence of MRSA in the state of Santa Catarina is extremely low. Isolation rates are between 4-8% between S. aureus and less than 2% among the agents of nosocomial infections. This fact explains that in a sample collected during nearly three years in the largest cities in the state, only 124 strains have been used.

Infectious processes were found in 13.7% of blood cultures, 9.7% of surgical wounds, 4% of urine samples, 37.1% of lower respiratory tract infections, 19.4% of osteomyelitis cases, 1.6% of surveillance cultures, 3.2% of abscesses and 11.3% of other anatomic sites. Considering the origin of the samples, 17 (13.7%) were considered from the community and 107 (86.3%) from the hospital.

The profile of antimicrobial resistance among 124 clinical isolates of MRSA was evaluated. Resistance rates were the following: amikacin (35.5%), gentamicin (33.1%), chloramphenicol (12.9%), tetracycline (20.2%), ciprofloxacin (79%), norfloxacin (72.6%), sulfamethoxazole-trimethoprim (20.2%), clindamycin (75%) and erythromycin (81.5%). The antibiotic linezolid and tigecycline were all considered as susceptible. In evaluating the presence of beta-lactamase, 93 strains (75%) showed production of beta-lactamase.

To assess the susceptibility to glycopeptides, MIC was determined by Etest (Figure 1). Regarding vancomycin, seven isolates showed MIC of 3.0 μg/mL and were tested again by microdilution, and three showed MIC of 1.0 μg/mL and four 2.0 μg/mL, all categorized as sensitive. Teicoplanin only showed an isolate with MIC values above the susceptible category by CLSI (sensitive $\leq$ 8 μg/mL) but was considered likely because the microdilution MIC was 4 μg/mL.

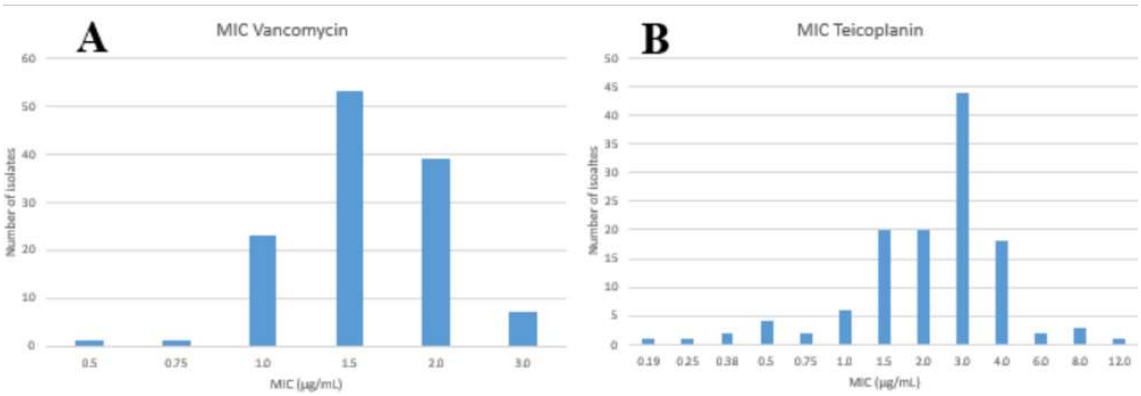

Figure 1 - MIC of the glycopeptides to 124 MRSA determined by Etest. In A, the values obtained for vancomycin. The seven isolates with MIC 3.0 µg/mL were considered susceptible because all determined by microdilution MICs were ≤ 2.0 µg/mL. In B, the results for teicoplanin. One isolate (MIC 12 µg/ml) was tested in microdilution MIC of 4.0 µg/mL (susceptible).

Daptomycin, an alternative to glycopeptides for the treatment of infections caused by MRSA, showed values within the sensitivity patterns (Figure 2), and four isolates showed MIC of 1.0 µg/mL (upper limit of sensitivity).

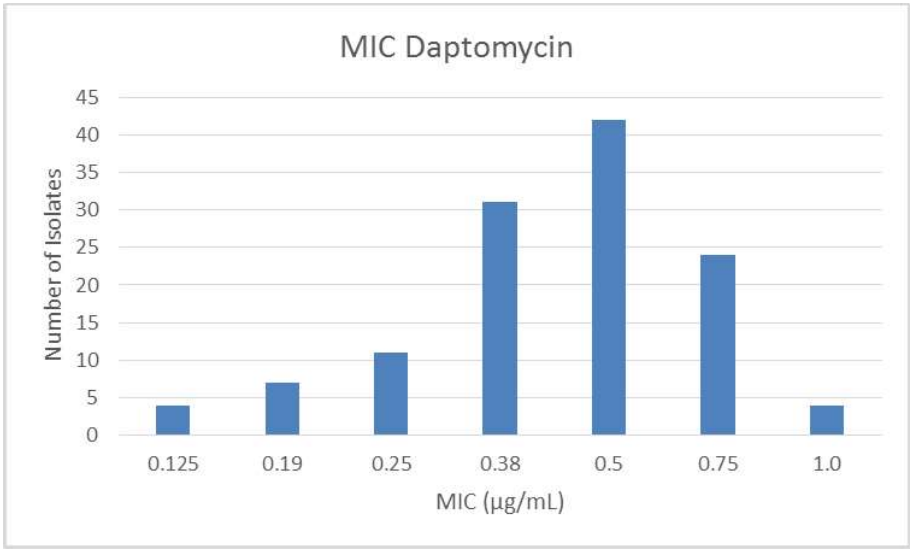

Figure 2 - MICs for daptomycin determined by Etest. All isolates were considered susceptible (MIC ≤ 1.0 µg/mL).

There was a heterogeneous distribution of SCC*mec* types. There was a predominance of type III, with sixty-seven isolates (54%), ten type I (8%), twenty-seven

type II (21.8%), 5 with type I and II, thirteen type IVa (10.5%), three type IVb (2.4%) and four type IVc (3.2%). Among the seventeen community isolates, only four showed SCC*mec* type IV: two IVa and two IVb. Among the other 13 isolates, eight had SCC*mec* type III and five had type II.

Of the 124 isolates, twenty-three (18.5%) had a positive D test, all confirmed by molecular tests, and twenty (87%) had *erm*A gene and 3 (13%) e*rm*C. Of the twelve (9.7%) isolates with resistance to erythromycin and susceptibility to clindamycin, those with a D negative test had no positive PCR reaction. Fifty-six (45.1%) isolates with resistance to both antimicrobials and with an inapplicable D test were considered to have a constitutive resistance mechanism and were not tested by PCR. Only thirty-three (26.6%) were susceptible to both antibiotics.

With respect to PFGE profiles, twenty-six pulsotypes (Figure 3) were found to have two or more isolates. They were considered clonal isolates that showed greater genetic similarity (80%), according to the criteria established by Tenover (1995). There was no dominant profile, and two pulsotypes were found with five isolates (CC6 and CC9); despite having SCC*mec* type III, the pulsotypes did not have an epidemiological relationship. A total of seventy-two (58%) isolates could be classified into any of the twenty-six clones, and fifty-two (42%) could not be classified into any clone (Table 1).

Table 1 - Epidemiological and antibiotic susceptibility characteristics of the 26 pulsotypes.

Pulsotypes	Isolates	Time	Source	City	Resistance	SCC*mec*
1	SI2, SI8	2-4/09	HOS	BLU	ER, CL, SU, CIP, N0, TE, CLO, AM, GE	II
2	SI10, SI29, L79	5/09 – 1/12	HOS	BLU, FLO	ER, CL, CIP, NO	II
3	L35, L77	9/10 – 12/11	HOS	FLO	ER, CL, CIP, NO	II
4	SI3, SI14	2/09 – 4/10	COM	BLU	ER, CL, CIP, NO	II
5	SI15, SI30	4/10 – 1/12	HOS	BLU	ER, CL, CIP, NO	II
6	SI1, SI5, SI9, L58, L86	1/09 – 3/12	HOS	BLU, FLO	ER, CL, CIP, NO	III
7	L40, L61, L62	10/10 – 4/11	HOS	FLO	ER, CL, CIP, NO	II
8	SI16, SI28, L72	5/10 – 11/11	HOS	BLU, FLO	CIP, NO	IVa
9	L22, L32, L46, L49, L81	7/10 – 1/12	HOS	FLO	ER, CL, CIP, NO	III
10	L39, L47, L48	8/10 – 11/10	HOS	FLO	ER, CL, CIP, NO	III
11	L55, L66, L88	6/11 – 5/12	HOS	FLO	ER, CL, CIP, NO	III
12	SI25, L27, L94	2/10 – 10/12	COM, HOS	BLU, FLO	ER, CL, CIP, NO	IVa
13	L5, L75	8/08 – 11/11	COM, HOS	FLO	-	IVa
14	L19, L25, L59	7/10 – 2/11	HOS	FLO	ER, CL, CIP, NO	IVa
15	L91, L93	7/12 – 10/12	HOS	FLO	ER, CL, CIP, NO	IVb
16	L20, L50	7/10 – 11/10	HOS	FLO	ER, CL, SU, CIP, NO, AM, GE	III
17	L24, L26, L28, L29	7/10 – 8/10	HOS	FLO	ER, CL, CIP, NO, AM, GE	I
18	L12, L21, L70	12/09 – 6/11	HOS	FLO	ER, CL, CIP, NO, CLO, AM, GE	III
19	L13, L14, L17	1/10 – 5/10	COM, HOS	FLO	ER, CL, SU, CIP, NO, TE, AM, GE	III
20	L3, L6	7/08 – 1/09	HOS	FLO	ER, CL, SU, CIP, NO, TE, CLO, AM, GE	III
21	L43, L51	10/10 – 11/10	HOS	FLO	ER, CL, SU, CIP, NO, AM, GE	III
22	SI19, L67	8/10 – 1/11	COM, HOS	BLU, FLO	ER, CL, CIP, NO	III
23	L2, L4	5/08 – 6/08	COM, HOS	FLO	-	IVa
24	L44, L63	10/10 – 7/11	HOS	FLO	ER, CL, CIP, NO, AM, GE	III
25	L54, L69, L92	5/11 – 8/12	HOS	FLO	ER, CL, SU, CIP, NO, TE, CLO, AM, GE	II
26	L36, L41, L42, L80	9/10 – 1/12	HOS	FLO	ER, CL, CIP, NO	II

HOS: hospital; COM: Community; BLU: Blumenau; FLO: Florianópolis; ER: erythromycin; CL: clindamycin; SU: trimethoprim/sulfamethoxazole; CIP: ciprofloxacin; NO: norfloxacin; TE; tetracycline; CLO: chloramphenicol; AM; amikacin; GE: gentamycin.

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206 HOS: hospital; COM: Community; BLU: Blumenau; FLO: Florianópolis; ER:
207 erythromycin; CL: clindamycin; SU: trimethoprim/sulfamethoxazole; CIP:
208 ciprofloxacin; NO: norfloxacin; TE; tetracycline; CLO: chloramphenicol; AM;
209 amikacin; GE: gentamycin.
210

211 **DISCUSSION**

212

213 The low prevalence of MRSA in the state of Santa Catarina is comparable to
214 rates found in Scandinavian countries[19,2]. In a report published by the World Health
215 Organization (WHO), several other European countries have national surveillance data
216 with very low rates of MRSA: Denmark (1.2%), Estonia (1.7%), Finland (2.8%),
217 Iceland (2.8%), Netherlands (1.4%), Norway (0.3%) and Sweden (0.8%)[21]. Santa
218 Catarina indices differ greatly from Brazil, which has MRSA rates of 29%[22]. These
219 countries may have succeeded in maintaining low MRSA rates because of effective
220 search-and-destroy policies and/or controlled antibiotic overuse[23]. These policies are not
221 practiced in Santa Catarina, which could be a cause of the difference in the prevalence
222 data between the data found in Santa Catarina and those found in the rest of Brazil.

223 Among the anatomical sites, there was a predominance of isolates in lower
224 respiratory tract infections, osteomyelitis, and bloodstream infections. These data are

quite different from those found in studies with similar characteristics, which demonstrate a predominance of bloodstream infections (39%)[24] or infections of skin and soft tissue (61.5%)[25].

Compared to the antimicrobial surveillance in the multicenter study SENTRY[1], resistance rates in this study were lower: ciprofloxacin (91.4 - 79%), tetracycline (46.7 - 20.2%), trimethoprim-sulfamethoxazole (68.1 - 20.2%), clindamycin (87.9 - 75%) and erythromycin (94 - 81.5%). A possible explanation for the large differences in rates of susceptibility could be due to the epidemiological profile. Cavalcante et al proposed phenotypic markers associated with SCC*mec* types. Resistance to tetracycline and trimethoprim-sulfamethoxazole may be associated with SCC*mec* type III, in which 100% resistance was found with both drugs. SCC*mec* type IV has absolute susceptibility, with only 2% of the isolates resistant to trimethoprim-sulfamethoxazole and 100 % susceptibility to tetracycline[26]. Therefore, there is a large predominance of SCC*mec* type III, which justifies why the resistance values in our study are lower than those found in the rest of Brazil.

The presence of beta-lactamase may result in unusual phenotypes known as BORSA (Borderline Oxacillin Resistant *Staphylococcus aureus*). These bacteria, despite lacking the *mec*A gene, may be resistant to oxacillin and are phenotypically characterized as MRSA[27]. They may also be responsible for elevated MICs of oxacillin. After therapy with beta-lactams, they may induce the development of vancomycin resistance, a phenotype called β-lactam antibiotics-induced vancomycin resistant *S. aureus* (BIVR)[28].

The values of MICs for antimicrobial vancomycin, teicoplanin and daptomycin are all within the normal range. Although the phenotype of resistance to these drugs is uncommon[29], 31.45% of the isolates showed MIC of 2.0 µg/ml for vancomycin, which

may suggest the development of a phenomenon of an upward trend for MICs for vancomycin, called "MIC creep"[30].

The isolates demonstrated higher prevalence of gene *erm*A than *erm*C. The D test showed a methodology capable of detecting all isolates with inducible clindamycin resistance. Therefore, the test is easy to perform and inexpensive and is clinical relevant because it reduces the risk of inappropriate antibiotics[31].

In our study, there was a high prevalence of SCC*mec* type III (54%), and its clone is called Brazilian epidemic clone (BEC) according to national epidemiology. In 2010, a study conducted at the Clinical Hospital of Porto Alegre, a high prevalence of SCC*mec* type III was detected (49%)[32], similar to the percentage found in our study. In 2005, in contrast to the local epidemiology, a study conducted at the Clinical Hospital of São Paulo showed a 65% MRSA prevalence among hospital SCC*mec* type IV[33].

Twenty-six pulsotypes were detected with a wide variety of mobile genetic elements, and there was no substantial prevalence of a particular clone. As the cities of Florianopolis and Blumenau are separated by approximately 120 kilometers and the isolates were collected during a period of three years, there was no significant epidemiological relationship between clonal complexes.

CONCLUSIONS

Although we identified the SCC*mec* that were present and evaluated the presence of clonal complexes, the present study did not provide consistent scientific evidence for the low prevalence of MRSA in Santa Catarina, which was very different from the data found in other Brazilian states and some European countries.

We conclude that the epidemiological profile is similar to that found in the rest of Brazil, with a predominance of SCC*mec* type III, but the resistance rates were lower than those found in the rest of Brazil.

A wide variety of complexes with clonal isolates were detected without evidence of clonal spread, and a small amount of high genetic diversity was found.

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VIII. DISCUSSÃO GERAL

Foram utilizadas amostras de duas das três cidades mais populosas do Estado: Florianópolis e Blumenau. A cidade de Joinville não foi incluída no estudo por motivos logísticos. Considerando a cidade de Florianópolis, os isolados foram gentilmente cedidos pelo laboratório Santa Luzia, sob supervisão de sua gerente geral, Cássia Zoccoli. O laboratório Santa Luzia é o maior laboratório do Estado, reconhecido nacional e internacionalmente pelo seu padrão de qualidade. Segundo dados do próprio laboratório, foram realizadas entre janeiro e setembro de 2012 5.673 hemoculturas, com 13.187 frascos. Deste contingente de amostras, apenas 8 MRSA foram isolados. Considerando dados publicados pelo SENTRY (GALES et al., 2009), a taxa de isolamento de *S. aureus* em hemoculturas é de 20,2 % e o percentual de MRSA de 31 %. Imaginado um percentual baixo de positividade de hemoculturas (10 %) e levando em conta os percentuais encontrados pelo SENTRY até 2008, poderiam ter sido isolados 81 MRSA, ou seja, dez vezes mais do que fora obtido.

Considerando a cidade de Blumenau, terceira maior do Estado, com aproximadamente 300.000 habitantes, as amostras foram obtidas no laboratório Santa Isabel que, apesar de situado fisicamente dentro do hospital, atende também pacientes ambulatoriais. O Hospital Santa Isabel é um hospital de médio porte, com 280 leitos, de alta complexidade, pois é referência nacional em transplante de rim, fígado e pâncreas.

À medida que os estudos, tanto de resistência como de epidemiologia, começaram a ser realizados, verificamos que os perfis encontrados em Santa

Catarina não diferem muito de outros estudos brasileiros, apenas aparecem em número menor. As taxas de resistência foram semelhantes aos estudos multicêntricos (GALES et al., 2009; JONES et al., 2013), assim como outros estudos locais (REITER et al., 2010; TEIXEIRA et al., 2012). Do ponto de vista molecular essa realidade se repete, sendo que houve um predomínio do SCC*mec* do tipo III (hospiatalar) e não houve evidência de disseminação clonal, visto que foram encontrados vinte e seis clones, com no máximo cinco isolados.

Para caracterização dos clones, foi utilizada a técnica de eletroforese de campo pulsado (PFGE). Consiste na utilização de DNA genômico digerido por enzimas de restrição (*Sma*I). É uma técnica considerada *gold standard* para caracterização de clones de *S. aureus* (TENOVER, 2003; FRICKMANN et al., 2012). Apresenta um perfil comparativo que permite avaliar a similaridade genética e, de acordo com critérios prévios, estabelecer um limite de 80 % para considerar dois isolados como geneticamente relacionados (TENOVER et al., 1995). A alta diversidade genética, visualizada com o achado de quatro tipos diferentes de SCC*mec*, com muitos complexos clonais minoritários, pode estar associado à baixa prevalência. Porém estudos epidemiológicos mais detalhados necessitariam ser realizados para chegar à tal conclusão.

Em países nos quais as taxas de isolamento de MRSA são baixas (FARIA et al., 2005; ELSTROM et al., 2012), comparáveis com as encontradas neste estudo, as explicações encontradas para tais taxas são as políticas governamentais, principalmente no controle de uso de antimicrobianos, detecção precoce de mecanismos de resistência e programas contínuos de vigilância epidemiológica. Não seria prudente afirmar que não encontramos

política semelhante em Santa Catarina, fato que dificulta ainda mais o entendimento do fenômeno ocorrido.

Por mais contraditório que possa parecer, apesar da prevalência de MRSA ser muito baixa, a prevalência de hVISA é semelhante às encontradas em muitos outros países, como na Australia (9,4 %) (CHARLES et al., 2004), United States (8,3 %) (RYBAK et al., 2008), Israel (6 %) (MAOR et al., 2007), France (11 %) (GARNIER et al., 2006).

Se considerarmos que um dos principais fatores de risco para o desenvolvimento do fenótipo hVISA é o uso prévio de glicopeptídeos (APPELBAUM, 2007; VAN HAL; FOWLER JR, 2013; HOWDEN; PELEG; STINEAR, 2014), novamente encontramos uma situação inusitada: se a taxa de MRSA é baixa, apesar do isolamento de *S. aureus*, tecnicamente não está indicado o uso de glicopeptídeo em isolado sensível à meticilina (MSSA), como encontramos uma prevalência de 9,7 % (semelhante à vários estudos internacionais) de hVISA? Infelizmente também não temos uma resposta para essa questão.

Os isolados hVISA apresentam características bem peculiares: são instáveis, heterogêneos, não detectados pelos métodos usuais (disco difusão e microdiluição em caldo) e usualmente apresentam variações na morfologia colonial (*small colony variants*), podendo confundir o microbiologista, pois um cultivo puro pode sugerir uma contaminação (HOWDEN et al., 2010). Por serem encontradas numa fração minoritária da população bacteriana (1 para cada 10^6 células), torna-se necessária a presença de um grande inóculo bacteriano para detecção do fenótipo.

Vários marcadores moleculares tem sido propostos para facilitar a detecção de hVISA (BISCHOFF; BERGER-BACHI, 2001; FINAN et al., 2001; SAKOULAS et al., 2002; NISHI et al., 2004; SAKOULAS et al., 2005; SEIDL et al., 2006; JANSEN et al., 2007; HOWDEN et al., 2008; RENZONI et al., 2009; MATSUO et al., 2011; CAFISO et al., 2012; MATSUO et al., 2013). Por se tratar de um mecanismo de resistência heterogêneo, com vários genes envolvidos, ainda não temos um marcador confiável. Dessa forma, um diagnóstico precoce e preciso deve ser realizado, a fim de evitar o uso inapropriado de glicopeptídeos.

Como hVISA necessita condições diferenciadas de nutrição e tempo de incubação, foram propostos testes fenotípicos que utilizam meios enriquecidos e com incubação prolongada (48 horas) (SATOLA et al., 2011; VAN HAL et al., 2011). Tais metodologias requerem procedimentos especiais, que dificilmente podem ser aplicados para todas as amostras clínicas de *S. aureus*, devido à sua complexidade e, principalmente, aumento dos custos (VAN HAL et al., 2011).

A vancomicina é um fármaco com alto peso molecular e que desde 2009 teve seus pontos de corte para disco difusão retirados do CLSI (CLSI, 2009). Com isso, os laboratórios de pequeno e médio porte tiveram dificuldade de testar a susceptibilidade à tal agente, pois os métodos de determinação da concentração inibitória mínima ainda não são uma realidade em nosso País, devido ao custo.

Associando os problemas de custo e conhecimento, podemos sugerir o motivo pelo qual as infecções causadas por hVISA não sejam detectadas e até mesmo negligenciadas em nosso País. Torna-se fundamental dessa maneira

propor metodologias simples, baratas e que possam ser aplicadas na maioria dos laboratórios.

Um bom exemplo de metodologia acessível é a pré-difusão. Trata-se de uma técnica descrita há muitos anos atrás, que pode ser utilizada para testar a susceptibilidade à antibacterianos que apresentam um elevado peso molecular e, consequentemente, uma menor difusão em ágar. Poucos trabalhos já foram publicados utilizando a pré-difusão para *S. aureus*, demonstrando sua utilidade (NIELSEN, CASALS, 2005; KATZ; LUPERCHIO; THORNE, 2008). Utiliza o princípio de um aumento no tempo da difusão, permitindo que o agente consiga se difundir pelo meio de maneira adequada, produzindo resultados fidedignos.

Avaliando a capacidade da pré-difusão em triar os possíveis isolados hVISA, verificamos que, apesar de estar longe do ideal, apresentou resultados semelhantes aos outros testes utilizados com este propósito (SATOLA et al., 2011; VAN HAL et al., 2011). Demonstrou um valor preditivo negativo de 97,1 %, podendo sugerir como um possível teste de triagem mas que, devido ao baixo valor preditivo positivo, deve necessariamente ser confirmado com metodologia de referência. Mesmo que não possa ser confirmado, devido às características do Brasil (dimensão continental e com dificuldades financeiras), pode ser utilizado para sugerir ao clínico que a terapêutica com vancomicina está associada com falha terapêutica. Outras drogas podem ser utilizadas no tratamento (VARDAKAS et al., 2012), como a linezolida, daptomicina, tigeciclina, clindamicina, dependendo obviamente da susceptibilidade do microrganismo.

Um dos principais objetivos deste trabalho foi de avaliar as metodologias utilizadas para detecção de hVISA. As três metodologias fenotípicas (Etest

GRD, macro Etest e ágar screening com 4 µg/mL de vancomicina) foram testadas, além da pré-difusão. Nenhuma apresentou resultados satisfatórios, que pudessem motivar sua indicação como único teste a ser utilizado (KHATIB et al., 2011). A sugestão é que sejam utilizadas em associação com informações pré-analíticas, como: sítio anatômico, uso prévio de vancomicina, co-morbidades (CASAPAO et al., 2013). Dados microbiológicos também são de suma importância, como a CIM e presença de variação na morfologia colonial (HOWDEN et al., 2010).

Fica evidente a dificuldade em realizar o diagnóstico de infecção causada por hVISA, evidenciando um grande desafio para o laboratório de microbiologia clínica. É fundamental, dessa forma, conhecer os dados epidemiológicos locais a fim de guiar uma antibioticoterapia empírica de qualidade, diminuindo a morbidade e a mortalidade associadas às infecções por tais micro-organismos (YAMALI et al., 2011).

Além dos testes propostos, alguns autores sugerem a determinação da CIM pela metodologia de Etest. Como tal método tende a super estimar as CIMs (PAIVA et al., 2010), parece estar relacionada de forma mais direta com a falha terapêutica, pois os pontos de corte adotados pelo CLSI e EUCAST (≤ 2 µg/mL) tem sido associados a falha terapêutica. Essa foi a principal razão para utilizarmos os dados das CIMs determinadas por Etest pois, apesar de termos realizado também a microdiluição em caldo, buscamos ferramentas que pudessem estar mais relacionadas à clínica, fundamentadas em metanálise e estudos científicos de grande impacto (HORNE et al., 2009; MAOR et al.,2009; PITZ et al., 2011; PARK et al., 2012; VAN HAL; LODISE; PATERSON, 2012; CASAPAO et al., 2013; VAN HAL; PATERSON, 2013; WANG et al., 2013;).

Uma das principais limitações do estudo foi que não tivemos acesso aos desfechos clínicos. Por se tratar de um estudo retrospectivo, no qual os laboratórios que prestam serviço às instituições hospitalares disponibilizavam os isolados, não foi possível verificar em quais pacientes houve falha terapêutica. Da mesma forma, não tivemos acesso à informação de antibioticoerapia prévia com vancomicina e/ou agentes beta-lactâmicos, que comprovadamente são fatores de risco para desenvolvimento de infecções por hVISA.

O conhecimento da epidemiologia molecular da região também é muito importante. No caso dos isolados hVISA, que são instáveis e geralmente necessitam de um fator predisponente, não era esperado que houvesse uma disseminação clonal. O gasto metabólico para manutenção de um espessamento de parede celular não é transmitido às gerações subsequentes da bactéria quando na ausência de um agente indutor, no caso a vancomicina. Verifica-se mesmo no laboratório que, se repicada por três ou quatro vezes, a bactéria acaba perdendo essa característica, diminuindo os valores das CIMs.

Apesar disso, foram encontrados entre os doze isolados hVISA detectados, três clones. Apenas dois isolados demonstraram alguma relação epidemiológica, pois foram detectados com menos de dois meses de diferença numa mesma instituição. Os outros isolados, apesar de uma similaridade genética, apresentaram uma significativa diferença temporal e/ou geográfica.

Como o mecanismo de resistência necessita do uso prévio e contínuo de vancomicina, requerendo um balanço energético favorável, é instável, não apresentando relatos de transmissão genética horizontal, não tendo sido associado a clones majoritários.

Os estudos com os isolados irão prosseguir, o próximo passo será verificar qual o clone (*sequence type*) de cada isolado hVISA através da técnica de MLST e, dessa forma, construir uma árvore filogenética. Talvez com tais informações possamos realizar inferências estatísticas e epidemiológicas mais precisas a respeito da disseminação de hVISA em Santa Catarina.

IX. REFERÊNCIAS

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Staphylococcus aureus (GISA), in Particular, Heterogeneous GISA. *Journal of Clinical Microbiology*, v. 46, n. 9, p. 3042-3047, 2008.

X. CONCLUSÕES

- Os MRSA isolados em Santa Catarina tem, tanto do ponto de vista de susceptibilidade aos antimicrobianos como de perfis epidemiológicos, semelhança com aqueles descritos no Brasil;
- Não houve evidência de disseminação clonal, visto que foram detectados 26 clones minoritários;
- Os testes de triagem para hVISA apresentaram sensibilidade e especificidade variáveis, indicando que sejam utilizados associados, a fim de aumentar a acurácia diagnóstica. De acordo com os dados epidemiológicos locais, cada laboratório deve escolher a metodologia mais indicada para detecção de hVISA, assim como criar um fluxograma de processamento destas amostras;
- O teste de pré-difusão pode ser uma metodologia útil na detecção de hVISA, pois é simples, barata e prática, apresentando taxas de sensibilidade e especificidade semelhantes aos outros testes fenotípicos utilizados;
- A incidência de hVISA no Estado é semelhante à descrita em outros países. O conhecimento do perfil epidemiológico, variáveis pré-analíticas e dados clínicos podem auxiliar na terapêutica empírica das infecções graves causadas por *S. aureus*;
- Não houve disseminação clonal preponderante entre os isolados hVISA, pois foram detectados vários perfis diferentes, mas sem relação epidemiológica entre si. As taxas de resistência aos antimicrobianos foram semelhantes às descritas para os isolados MRSA.

XI. PERSPECTIVAS FUTURAS

- Análise comparativa do genoma de isolados clínicos de *Staphylococcus aureus* com hetero-resistência à vancomicina (hVISA) e papel dos genes *vra*SR e *gra*SR na diminuição da susceptibilidade à vancomicina, em parceria com a Universidade Federal de Santa Catarina;
- Avaliação dos clones circulantes hVISA através da técnica de Multilocus Sequence Typing (MLST);
- Estudo da resistência heterogênea à vancomicina em *Staphylococcus aureus* através da genética comparativa de subpopulações de um isolado (*single cell genomic*), em parceria com a Universidade de São Paulo;
- Sequenciamento do genoma completo dos doze isolados hVISA, em parceria com a Universidade de São Paulo e o Instituto Evandro Chagas, Pará;
- Avaliação de isolados clínicos hVISA e cepas de referência através de espectrometria de massa (MALDI-TOF), para detecção de possíveis marcadores de diminuição de susceptibilidade à vancomicina;
- Implantação no laboratório de microbiologia clínica da Universidade Regional de Blumenau (FURB) de um centro de detecção e vigilância epidemiológica de bactérias multi-resistentes no estado de Santa Catarina.

XII. ANEXOS

ANEXO A. CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA DA UFCSPA

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Epidemiologia molecular e perfil de sensibilidade aos antimicrobianos de Staphylococcus aureus resistente a meticilina (MRSA)isolados de hospitais em Santa Catarina

Pesquisador Responsável Pedro Alves D' Azevedo **Parecer** 1804/12

Data da Versão 12/04/2012 **Cadastro** 974/12 **Data do Parecer** 16/08/2012

Grupo e Área Temática III - Projeto fora das áreas temáticas especiais

Objetivos do Projeto
Caracterizar fenotipicamente e genotipicamente as amostras de MRSA circulantes em Santa Catarina de 2010 a 2012.

Sumário do Projeto

Itens Metodológicos e Éticos	Situação
Título	Adequado
Autores	Adequados
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não
Aprovação no país de origem	Não necessita
Local de Realização	Própria instituição
Outras instituições envolvidas	Sim
Condições para realização	Adequadas

Comentários sobre os itens de identificação

Os isolados bacterianos para o estudo foram doados pelo Laboratório Santa Luzia, de Florianópolis e do Laboratório Santa Isabel, de Blumenau.

Introdução	Adequada

Comentários sobre a introdução

Objetivos	Adequados

Comentários sobre os Objetivos

Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total 150 Local
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Não
Seleção eqüitativa dos indivíduos participantes	Não se aplica
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Não se aplica
Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Não utiliza
Monitoramento da segurança e dados	Não necessário
Avaliação dos dados	Adequada - qualitativa
Privacidade e confidencialidade	Adequada
Termo de Consentimento	Adequado
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Cronograma	Comentário
Data de início prevista	
Data de término prevista	
Orçamento	Adequado
Fonte de financiamento externa	Não

Página 1-2

Comentários sobre o Cronograma e o Orçamento

Organizar o cronograma, pois o projeto, na sua fase experimental, está iniciando antes da aprovação do CEP.

Referências Bibliográficas	Adequadas

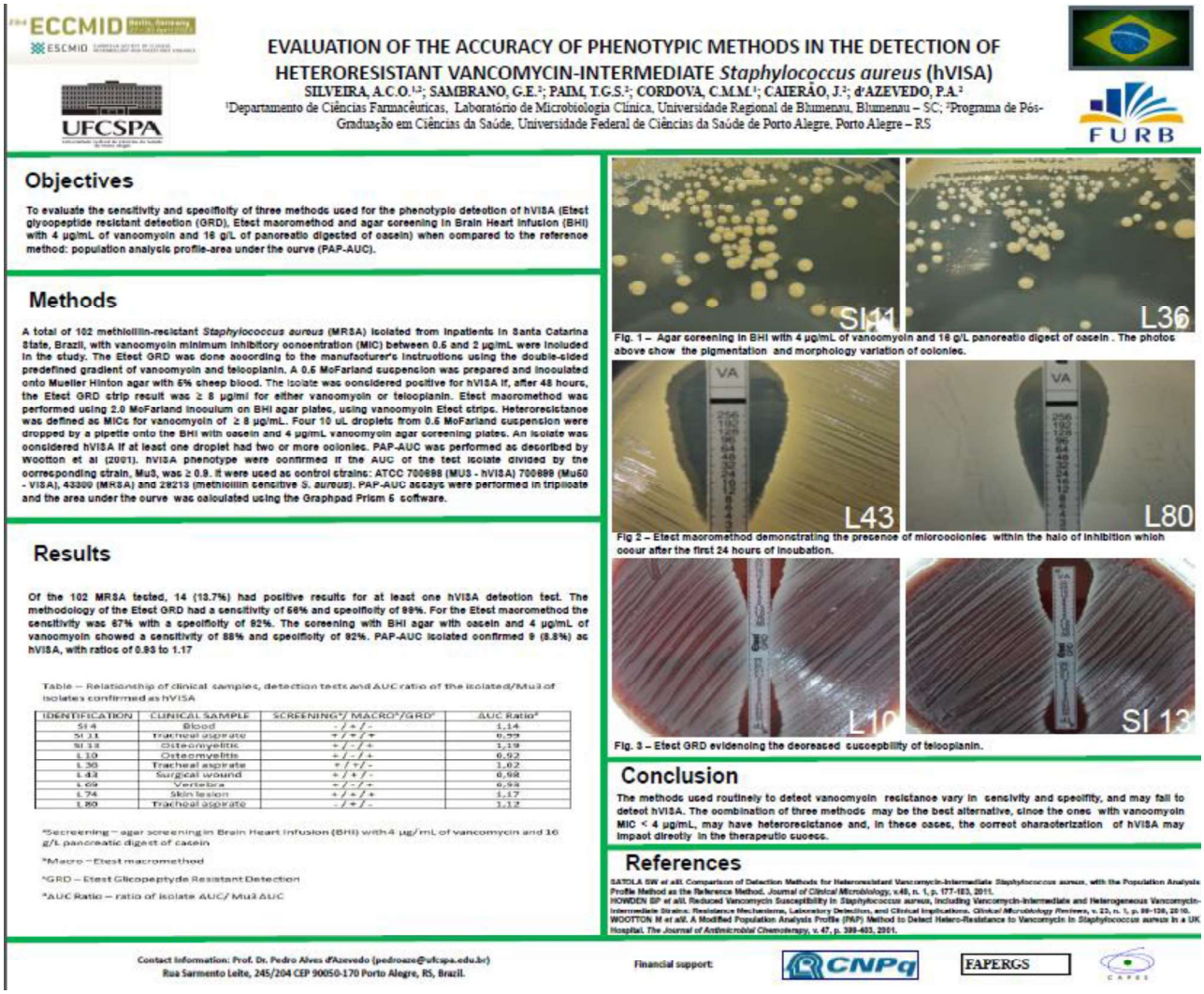
Comentários sobre as Referências Bibliográficas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

ANEXO B. RESUMOS APRESENTADOS EM CONGRESSOS INTERNACIONAIS



EVALUATION OF THE ACCURACY OF PHENOTYPIC METHODS IN THE DETECTION OF HETERORESISTANT VANCOMYCIN-INTERMEDIATE *Staphylococcus aureus* (hVISA)

SILVEIRA, A.C.O.[1,2]; SAMBRANO, G.E.[2]; PAIM, T.G.S.[2]; CORDOVA, C.M.M.[1]; CAIERÃO, J.[2]; d'AZEVEDO, P.A.[2]

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Objectives

To evaluate the sensitivity and specificity of three methods used for the phenotypic detection of hVISA (Etest glycopeptide resistant detection (GRD), Etest macromethod and agar screening in Brain Heart Infusion (BHI) with 4 µg/mL of vancomycin and 16 g/L of pancreatic digested of casein) when compared to the reference method: population analysis profile-area under the curve (PAP-AUC).

Methods

A total of 102 methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from inpatients in Santa Catarina State, Brazil, with vancomycin minimum inhibitory concentration (MIC) between 0.5 and 2 µg/mL were included in the study. The Etest GRD was done according to the manufacturer's instructions using the double-sided predefined gradient of vancomycin and teicoplanin. A 0.5 McFarland suspension was prepared and inoculated onto Mueller Hinton agar with 5% sheep blood. The isolate was considered positive for hVISA if, after 48 hours, the Etest GRD strip result was ≥ 8 µg/ml for either vancomycin or teicoplanin. Etest macromethod was performed using 2.0 McFarland inoculum on BHI agar plates, using vancomycin Etest strips. Heteroresistance was defined as MICs for vancomycin of ≥ 8 µg/mL. Four 10 uL droplets from 0.5 McFarland suspension were dropped by a pipette onto the BHI with oxacin and 4 µg/mL vancomycin agar screening plates. An isolate was considered hVISA if at least one droplet had two or more colonies. PAP-AUC was performed as described by Wootton et al (2001). hVISA phenotype were confirmed if the AUC of the test isolate divided by the corresponding strain, Mu3, was ≥ 0.9. It were used as control strains: ATCC 700698 (MU3 - hVISA) 700699 (Mu50 - VISA), 43300 (MRSA) and 29213 (methicillin sensitive *S. aureus*). PAP-AUC assays were performed in triplicate and the area under the curve was calculated using the Graphpad Prism 6 software.

Results

Of the 102 MRSA tested, 14 (13.7%) had positive results for at least one hVISA detection test. The methodology of the Etest GRD had a sensitivity of 68% and specificity of 99%. For the Etest macromethod the sensitivity was 87% with a specificity of 92%. The screening with BHI agar with oxacin and 4 µg/mL of vancomycin showed a sensitivity of 88% and specificity of 92%. PAP-AUC isolated confirmed 9 (8.8%) as hVISA, with ratios of 0.93 to 1.17

Table – Relationship of clinical samples, detection tests and AUC ratio of the isolated/Mu3 of isolates confirmed as hVISA

IDENTIFICATION	CLINICAL SAMPLE	SCREENING*/ MACRO*/GRD*	AUC Ratio*
SI 4	Blood	- / + / -	1,14
SI 11	Tracheal asprate	+ / + / +	0,99
SI 13	Osteomyelitis	+ / - / +	1,19
L 10	Osteomyelitis	+ / - / +	0,92
L 36	Tracheal asprate	+ / +/ -	1,02
L 43	Surgical wound	+ / + / -	0,98
L 69	Vertebra	+ / - / +	0,93
L 74	Skin lesion	+ / + / +	1,17
L 80	Tracheal asprate	- / + / -	1,12

*Screening – agar screening in Brain Heart Infusion (BHI) with 4 µg/mL of vancomycin and 16 g/L pancreatic digest of casein

*Macro – Etest macromethod

*GRD – Etest Glicopeptyde Resistant Detection

*AUC Ratio – ratio of isolate AUC/ Mu3 AUC

Fig. 1 – Agar screening in BHI with 4 µg/mL of vancomycin and 16 g/L pancreatic digest of casein . The photos above show the pigmentation and morphology variation of colonies.

Fig 2 – Etest macromethod demonstrating the presence of microcolonies within the halo of inhibition which occur after the first 24 hours of incubation.

Fig. 3 – Etest GRD evidencing the decreased sucoeptibility of teicoplanin.

Conclusion

The methods used routinely to detect vancomycin resistance vary in sensivity and specificity, and may fail to detect hVISA. The combination of three methods may be the best alternative, since the ones with vancomycin MIC < 4 µg/mL, may have heteroresistance and, in these cases, the correct characterization of hVISA may impact directly in the therapeutic sucess.

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WOOTTON M et all. A Modified Population Analysis Profile (PAP) Method to Detect Hetero-Resistance to Vancomycin in Staphylococcus aureus in a UK Hospital. *The Journal of Antimicrobial Chemotherapy*, v. 47, p. 399-403, 2001.

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Financial support: CNPq FAPERGS CAPES

EVALUATION OF THE ACCURACY OF PHENOTYPIC METHODS IN THE DETECTION OF HETERORESISTANT VANCOMYCIN-INTERMEDIATE *Staphylococcus aureus* (hVISA)

SILVEIRA, A.C.O.[1,2]; SAMBRANO, G.E.[2]; PAIM, T.G.S.[2]; CORDOVA, C.M.M.[1]; CAIERÃO, J.[2]; d'AZEVEDO, P.A.[2]

[1]Departamento de Ciências Farmacêuticas, Laboratório de Microbiologia, Universidade Regional de Blumenau, Blumenau – SC; [2]Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre – RS

Objectives: To evaluate the sensitivity and specificity of three methods used for the phenotypic detection hVISA (Etest glycopeptide resistant detection (GRD), Etest macromethod and agar screening in Brain Heart Infusion (BHI) with 4 µg/mL of vancomycin and 16 g /L pancreatic digest of casein) when compared to the reference method, population analysis profile-area under the curve (PAP-AUC). **Methods**:A total of 102 methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from hospitals in the state of Santa Catarina, Brazil, with vancomycin minimum inhibitory concentration (MIC) between 0.5 and 2 µg/mL. The Etest GRD was done according to the manufacturer's instructions using the double-sided predefined gradient of vancomycin and teicoplanin. A 0.5 McFarland suspension was prepared and swabbed onto Mueller Hinton agar with 5% sheep blood. The test isolate was considered positive for hVISA if, after 48 hours, the Etest GRD strip result was ≥ 8 µg/ml for either vancomycin or teicoplanin. Etest macromethod was performed using 2.0 McFarland inoculum on BHI agar plates, using vancomycin Etest strips. Heteroresistant was defined as MICs for vancomycin of ≥ 8 µg/mL. Four 10 uL droplets from 0.5 McFarland suspension were dropped by a pipette onto the BHI with casein and 4 µg/mL vancomycin agar screening plates. An isolate was considered hVISA if at least one droplet had two or more colonies. PAP-AUC was performed as described by Wootton et al. HVISA phenotype were confirmed to if the AUC of the test isolate divided by the corresponding strain, MU3, was ≥ 0.9. **Results:** Of the 102 MRSA tested, 14 (13.7%) had positive results for at least one as hVISA detection tests. The methodology of the Etest GRD had a sensitivity of 56% and specificity of 99%. As for the Etest macromethod sensitivity was 67% with a specificity of 92%. The screening with BHI agar with casein and 4 µg/mL of vancomycin showed a sensitivity of 88% and specificity of 92%. PAP-AUC isolated confirmed 9 (8.8%) as hVISA, with ratios of 0.93 to 1.17 (Table). **Conclusion:** The methods used routinely to detect vancomycin resistance vary in sensitivity and specificity, and may fail to detect hVISA. The combination of the three methods may be the best alternative, since the ones with vancomycin MIC < 4 µg/mL, may have hetero-resistance and, in these cases, the correct characterization of hVISA may impact directly in the therapeutic success.

Financial support: CNPq, FAPERGS

Table – Relationship of clinical samples, detection tests and AUC ratio of the isolated/Mu3 of isolates confirmed as hVISA

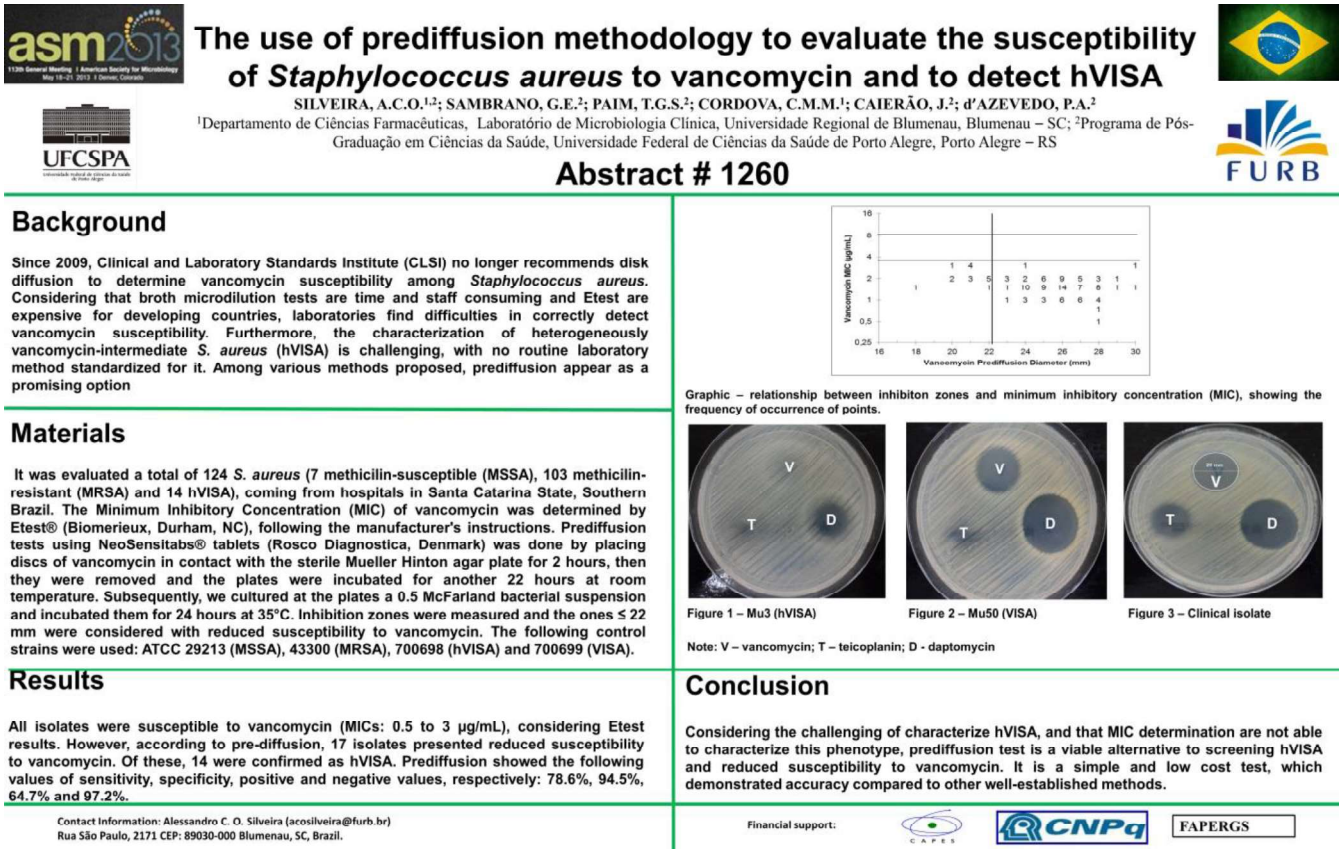
IDENTIFICATION	CLINICAL SAMPLE	SCREENING[a]/ MACRO[b]/GRD[c]	AUC Ratio[d]
SI 4	Blood	- / + / -	1,14
SI 11	Tracheal aspirate	+ / + / +	0,99
SI 13	Osteomyelitis	+ / - / +	1,19
L 10	Osteomyelitis	+ / - / +	0,92
L 36	Tracheal aspirate	+ / +/ -	1,02
L 43	Surgical wound	+ / + / -	0,98
L 69	Vertebra	+ / - / +	0,93
L 74	Skin lesion	+ / + / +	1,17
L 80	Tracheal aspirate	- / + / -	1,12

[a]Secreening – agar screening in Brain Heart Infusion (BHI) with 4 µg/mL of vancomycin and 16 g/L pancreatic digest of casein

[b]Macro –Etest macromethod

[c]GRD – Etest Glicopeptyde Resistant Detection

[d]AUC Ratio – ratio of isolate AUC/ Mu3 AUC



The use of prediffusion methodology to evaluate the susceptibility of *Staphylococcus aureus* to vancomycin and to detect hVISA

SILVEIRA, A.C.O.[1,2]; SAMBRANO, G.E.[2]; PAIM, T.G.S.[2]; CORDOVA, C.M.M.[1]; CAIERÃO, J.[2]; d'AZEVEDO, P.A.[2]

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Abstract # 1260

Background

Since 2009, Clinical and Laboratory Standards Institute (CLSI) no longer recommends disk diffusion to determine vancomycin susceptibility among *Staphylococcus aureus*. Considering that broth microdilution tests are time and staff consuming and Etest are expensive for developing countries, laboratories find difficulties in correctly detect vancomycin susceptibility. Furthermore, the characterization of heterogeneously vancomycin-intermediate *S. aureus* (hVISA) is challenging, with no routine laboratory method standardized for it. Among various methods proposed, prediffusion appear as a promising option

Materials

It was evaluated a total of 124 *S. aureus* (7 methicilin-susceptible (MSSA), 103 methicilin-resistant (MRSA) and 14 hVISA), coming from hospitals in Santa Catarina State, Southern Brazil. The Minimum Inhibitory Concentration (MIC) of vancomycin was determined by Etest® (Biomerieux, Durham, NC), following the manufacturer's instructions. Prediffusion tests using NeoSensitabs® tablets (Rosco Diagnostica, Denmark) was done by placing discs of vancomycin in contact with the sterile Mueller Hinton agar plate for 2 hours, then they were removed and the plates were incubated for another 22 hours at room temperature. Subsequently, we cultured at the plates a 0.5 McFarland bacterial suspension and incubated them for 24 hours at 35°C. Inhibition zones were measured and the ones ≤ 22 mm were considered with reduced susceptibility to vancomycin. The following control strains were used: ATCC 29213 (MSSA), 43300 (MRSA), 700698 (hVISA) and 700699 (VISA).

Results

All isolates were susceptible to vancomycin (MICs: 0.5 to 3 µg/mL), considering Etest results. However, according to pre-diffusion, 17 isolates presented reduced susceptibility to vancomycin. Of these, 14 were confirmed as hVISA. Prediffusion showed the following values of sensitivity, specificity, positive and negative values, respectively: 78.6%, 94.5%, 64.7% and 97.2%.

Graphic – relationship between inhibiton zones and minimum inhibitory concentration (MIC), showing the frequency of occurrence of points.

Figure 1 – Mu3 (hVISA)

Figure 2 – Mu50 (VISA)

Figure 3 – Clinical isolate

Note: V – vancomycin; T – teicoplanin; D - daptomycin

Conclusion

Considering the challenging of characterize hVISA, and that MIC determination are not able to characterize this phenotype, prediffusion test is a viable alternative to screening hVISA and reduced susceptibility to vancomycin. It is a simple and low cost test, which demonstrated accuracy compared to other well-established methods.

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Financial support: CAPES, CNPq, FAPERGS

The use of prediffusion methodology to evaluate the susceptibility of *Staphylococcus aureus* to vancomycin and to detect hVISA

SILVEIRA, ACO[1,2]; SAMBRANO, GE[2]; CAIERÃO, J[2]; CÓRDOVA, CMM[1]; d'AZEVEDO, PA[2].

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Background: Since 2009, Clinical and Laboratory Standards Institute (CLSI) no longer recommends disk diffusion to determine vancomycin susceptibility

among *Staphylococcus aureus.* Considering that broth microdilution tests are time and staff consuming and Etest are expensive for developing countries, laboratories find difficulties in correctly detect vancomycin susceptibility. Furthermore, the characterization of heterogeneously vancomycin-intermediate *S. aureus* (hVISA) is challenging, with no routine laboratory method standardized for it. Among various methods proposed, prediffusion appear as a promising option. **Materials:** It was evaluated a total of 124 *S. aureus* (103 methicilin-resistant (MRSA) and 14 hVISA), coming from hospitals in Santa Catarina State, Southern Brazil. The Minimum Inhibitory Concentration (MIC) of vancomycin was determined by Etest® (Biomerieux, Durham, NC), following the manufacturer's instructions. Prediffusion tests using NeoSensitabs® tablets (Rosco Diagnostica, Denmark) was done by placing discs of vancomycin in contact with the sterile Mueller Hinton agar plate for 2 hours, then they were removed and the plates were incubated for another 22 hours at room temperature. Subsequently, we cultured at the plates a 0.5 McFarland bacterial suspension and incubated them for 24 hours at 35°C. Inhibition zones were measured and the ones ≤ 22 mm were considered with reduced susceptibility to vancomycin. The following control strains were used: ATCC 29213 (MSSA), 43300 (MRSA) and 700698 (hVISA). **Results:** All isolates were susceptible to vancomycin (MICs: 0.5 to 3 µg/mL), considering Etest results. However, according to pre-diffusion, 17 isolates presented reduced susceptibility to vancomycin. Of these, 14 were confirmed as hVISA. Prediffusion showed the following values of sensitivity, specificity, positive and negative values, respectively: 78.6%, 94.5%, 64.7% and 97.2%. **Conclusion:** Considering the challenging of characterize hVISA, and that MIC determination are not able to

characterize this phenotype, prediffusion test is a viable alternative to screening hVISA and reduced susceptibility to vancomycin. It is a simple and low cost test, which demonstrated accuracy compared to other well-established methods.

Financial support: CNPq, FAPERGS

24th ECCMID Barcelona, Spain 10 - 13 May 2014

ESCMID EUROPEAN SOCIETY OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES

R170
Publication Only
Antimicrobials: Epidemiology of MRSA, VRE and other Gram-positives

Molecular epidemiology of *Staphylococcus aureus* with hetero-resistance to vancomycin (hVISA) in southern Brazil

A. Silveira[1], G. Cunha[1], B. Batista[1], J. Caierão[1], P. D'Azevedo[1]

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Objectives: To determine the epidemiological and molecular characteristics of twelve *Staphylococcus aureus* isolates presenting hetero-resistance to vancomycin (hVISA) in laboratories of two cities of Santa Catarina, southern Brazil. **Methods:** Epidemiological data including the city of isolation, health institution, and date of isolation were considered, as well as the associated clinical specimen. For molecular characterization, we analyzed the types of staphylococcal cassette chromosome (SCC*mec*), the presence of the *erm* gene (in cases of inducible clindamycin resistance profiles) and the genomic diversity of isolates, using pulsed field gel electrophoresis (PFGE). All isolates were characterized as methicillin-resistant (MRSA) through the use of cefoxitin disk, according to the recommendations of CLSI. The twelve isolates of *S. aureus* were previously confirmed as presenting hVISA using the technique of population analysis profile (PAP), and were collected between November 2009 and November 2012. The criteria used to consider an isolate as

community was the isolation of the bacteria in the first 48 hours of hospitalization. For determination of SCC*mec*, multiplex PCR was performed, identifying sub-types I to V. The search for *erm* gene was made by simplex PCR. **Results:** Among the isolates, nine were recovered in Florianopolis and three in Blumenau; eleven were associated to the hospital environment and one was isolated in community (Florianopolis). The anatomical sites of isolation included tracheal aspirate (n=5), osteomyelitis (n=4), surgical wound (n=1), skin lesion (n=1), and blood (n=1). Two isolates presented type I SCC*mec*, seven had type II, and two were positive for type III. It should be noted that one isolate presented two type of SCC*mec* (I and II) and two isolates were not typeable. The isolate from the community had type II SCC*mec*. Only three isolates showed inducible clindamycin resistance, all carrying gene *erm*A. Considering genetic variability, three clones were detected: the main one (clone A) composed of four isolates and two other clones (B and C) with two isolates each. In clone A, two isolates presented identical band patterns and they were related to the same hospital, with an interval of fifty-seven days between the isolation of both. The other isolates of this clone showed no epidemiological link between them, since they were isolated in different hospitals and had no temporal relationship. The other two clones showed no detectable epidemiological relationship. **Conclusion:** Contrary to expectations (type IV SCC*mec*), the communityassociated *S. aureus* showed type II SCC*mec*. hVISA recovered in Santa Catarina State, from 2009 to 2012 had, in general, heterogeneous genomic patterns by PFGE results, which is in accordance to the fact that these isolates had little or no

epidemiological relationship among them. Fitness required to maintain hVISA phenotype may explain the absence of clonal spread.

Financial support: CNPq, CAPES, FAPERGS

Isolate number	City	Institution	Clinical sample	SCC*mec*	Clone	Date
10	Florianópolis	Hospital A	Osteomyelitis	nt[a]	Non clonal	23/11/2009
36	Florianópolis	Hospital A	Tracheal aspirate	II	B	03/09/2010
43	Florianópolis	Hospital A	Surgical wound	III	C	14/10/2010
54	Florianópolis	Hospital A	Osteomyelitis	II	A	04/05/2011
69	Florianópolis	Hospital A	Osteomyelitis	II	A	01/07/2011
74	Florianópolis	Hospital B	Skin lesion	nt[a]	Non clonal	22/12/2011
80	Florianópolis	Clinic A	Tracheal aspirate	II	B	09/01/2012
84	Florianópolis	Hospital A	Tracheal aspirate	I	Non clonal	25/02/2012
92	Florianópolis	Hospital C	Tracheal aspirate	II	A	18/08/2012
SI4	Blumenau	Hospital D	Blood	I,II	A	28/09/2010
SI11	Blumenau	Hospital D	Tracheal aspirate	II	Non clonal	20/02/2011
SI13	Blumenau	Hospital D	Osteomyelitis	III	C	12/04/2011

[a]non typeable

ANEXO C. RESUMOS APRESENTADOS EM CONGRESSOS NACIONAIS

SILVEIRA, A. C. O.; AZEVEDO, P. A. Avaliação da Susceptibilidade à Teicoplanina Através da Metodologia de Disco Difusão em Isolados de *Staphylococcus aureus* Resistentes à Meticilina (MRSA). In: 41 Congresso Brasileiro de Análises Clínicas, 2014, Porto Alegre.

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ANEXO D. REGRAS PARA SUBMISSÃO: CANADIAN JOURNAL OF INFECTIOUS DISEASES & MEDICAL MICROBIOLOGY

THE CANADIAN JOURNAL OF
INFECTIOUS DISEASES &
MEDICAL MICROBIOLOGY
JOURNAL CANADIEN DES MALADIES INFECTIEUSES
ET DE LA MICROBIOLOGIE MEDICALE

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1. Kohl P, Day K, Noble D, et al. Cellular mechanisms of cardiac mechano-electric feedback in a mathematical model. Can J Cardiol 1998;14:111-9.

Books:

2. Svensson LG, Crawford ES. Cardiovascular and Vascular Disease of the Aorta. Toronto: WB Saunders Company, 1997:184-5.

Chapter in book:

3. Trehan S, Anderson JL. Thrombolytic therapy. In: Yusuf S, Cairns JA, eds. Evidence Based Cardiology. London: BMJ Books, 1998:419-44.

Web sites:

4. National Library of Medicine. Images from the History of Medicine. <www.ihm.nlm.nih.gov/> (Accessed January 5, 1999).

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ANEXO E. REGRAS PARA SUBMISSÃO: JOURNAL OF MEDICAL MICROBIOLOGY

1. Scope and Editorial Policy – Contents
1.1 Scope
1.1.1 General
The Editorial Board of the *Journal of Medical Microbiology* (JMM) wishes to publish excellent original scientific research papers or related original reviews and editorials, all of which should adhere to the following criteria. Subject matter should be within the ambit of medical microbiology, covering all types of micro-organism. Generally papers should be written from the perspective of microbiology rather than infectious disease. Basic research on the biology of pathogens, set in the context of infection or immunity, is acceptable. Host-centred topics, for example in host cell biology or immunology, will only be considered if there is a strong connection to a specific infection(s). Research findings should be novel, original and have a significant impact and are expected to go beyond purely descriptive or factual reporting of observations. For example, acceptable content might uncover underlying mechanisms or explanations of phenomena observed, or provide novel interpretation of observations. Descriptions of novel organisms associated with disease would be more suitable for *International Journal of Systematic and Evolutionary Microbiology*. If in doubt, the Editor-in-Chief will be pleased to advise authors about eligibility prior to submission, without prejudice to the acceptance or rejection of any papers subsequently submitted.

1.1.2 Types of paper
As of 5th September 2013 JMM no longer accepts Case Reports for submission. The Society for General Microbiology has launched a new Open Access online-only journal focussing solely on case reports. For further information regarding JMM Case Reports, please visit JMMCR. JMMCR is now accepting submissions at http://www.editorialmanager.com/jmmcr/
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- Models of infection
- Human and animal microbial ecology
- Correspondence

In reading the following guidelines, bear in mind that one full printed page comprises approximately 900 words of normal text, or an average of 500 words when tables and figures are taken into account.
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Correspondence. The Correspondence section is where readers of JMM can communicate their personal observations and opinions, useful methodologies, new theories or alternative interpretations of others' work. Only articles of a high level of interest to a wide range of readers of JMM will be considered. Reports of outbreaks should be dealt with as Case Reports. Correspondence items should be no more than 2 printed pages including references, with a maximum of 1 table or figure.
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1.2 Submission and publication requirements
1.2.1 Page and colour charges
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For references with ten or fewer authors, give the names of all authors in the form "Surname, Initials". For references with more than ten authors, list the first nine followed by "& other authors".
Sample journal references:
Cerdà-Cuéllar, M., Rosselló-Mora, R. A., Lalucat, J., Jofre, J. & Blanch, A. (1997). *Vibrio scophthalmi* sp. nov., a new species from turbot (*Scophthalmus maximus*). *Int J Syst Bacteriol* **47**, 58–61.
Pasta, F. & Sicard, M. A. (1996). Exclusion of long heterologous insertions and deletions from the pairing synapsis in pneumococcal transformation. *Microbiology* **142**, 695–705.
Sample journal reference for more than ten authors:
Tomb, J.-F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H.-P., Gill, S. & other authors (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature***388**, 539–547.
Sample reference to a whole book:
Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
Sample reference to a book chapter or section:
Romano, A. H. & Saier, M. H., Jr (1992). Evolution of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. I. Physiological and organismic considerations. In *The Evolution of Metabolic Function*, pp. 171–204. Edited by R. P. Mortlock. Boca Raton, FL: CRC Press.
References to websites
It is not practical to provide a generic example of a reference to a website. Essential items that must be provided are:

- an author(s) (which may be a company name or organization);
- a year of 'publication' (which may be the year that the site was last updated);
- the URL (web address) of the page;
- a page title (which will hopefully allow the page to be found using a search engine if the URL subsequently changes)

For a website that is frequently updated, it may be useful to provide the date that the site was accessed, particularly if specific information is quoted that may have changed when the article is read.>

Authors who use **EndNote** or **Reference Manager** can download the style for JMM by clicking on the links below:
EndNote
Reference Manager
Please note the following style points:

- References in the list must be given in alphabetical order, except for papers with three or more authors, which should be listed in chronological order after any other papers by the first author.
- References must include the title of the paper as well as both initial and final page numbers.
- Titles of journals should be abbreviated according to the system used by MEDLINE; no stops should be used after abbreviated words.
- References to books should include year of publication, title (in full), edition, editor(s) (if any), town of publication and publisher, in that order. When the reference is to a particular part of a book, the inclusive page numbers of the chapter or section and, if appropriate, chapter title must be given.
- Only papers accepted for publication but not yet published may be cited as 'in press' in the reference list, and the reference must include the name of the journal. Relevant papers cited as 'in press' should be included as supplementary files with the online submission. References to papers not yet accepted should be cited in the text as unpublished results, giving the surname(s) and initials of all the author(s). Such papers should not appear in the list of references.
- Permission must be obtained for any personal communications or citations of other workers' unpublished results.

2.5.10 Tables

These should be broadly comprehensible without reference to the text, but it is not necessary to repeat detailed descriptions of methods, etc. The symbols * † ‡ § ‖ ¶ # should be used for footnotes, rather than superscript letters or numbers. When results are expressed as percentages, the absolute value(s) corresponding to 100% must be stated. Statements of reproducibility should be included (see above). Tables should not be used to present results that can be described by a brief statement in the text.

2.5.11 Figures

This section outlines journal policy on figures. See these links for advice on preparing figures for inclusion as a PDF forsubmission and on the source files needed for publication.
Figures should not be used to present results that can be described by a brief statement in the text. The points outlined above for tables regarding comprehensibility, relative values and reproducibility also apply to figures and their legends. The inclusion of large amounts of tabular data in figures is discouraged and authors may be asked to move such data to the text or a separate table. Authors should be aware that after publication, tabulated data within figures are not accessible via online text searching. Where possible, please also supply line drawings, bar diagrams and sequence data in the original file format in which they were generated and/or as EPS (Encapsulated PostScript), PowerPoint or CorelDraw files. Do *not* supply as PostScript files as these cannot be used.
Figures must be referred to in the text as Fig. 1(a) **not** Fig. 1A or Figure 1(A) or as (Fig. 1a) **not** (Figure 1A). Multipart figures should be labelled (a), (b), etc., **not** (A), (B), etc.
Line drawings. These should be of a quality suitable for direct reproduction. The maximum printed size, including lettering and legends, is 176 x 235 mm. Line thicknesses and symbol sizes should be sufficient to allow for reduction. The preferred symbols for graphs are filled and open circles, squares, triangles or diamonds. Where possible, the same symbol should be used for the same quantity in different figures.
Bar diagrams. Simple bar diagrams reporting only a few values are usually unnecessary; the data can normally be given in a few lines of text. It is editorial policy not to publish bar diagrams with 'three-dimensional' bars unless there is a specific justification for their use.
Sequence data. Figures showing full gene sequences are not published, but selected sequence data, with appropriate annotation, may be published where there is justification. The layout of sequence figures should be designed to fit either the full width of the page (176 mm) or a single column (84 mm). For adequate legibility, the height of the characters should be not less than 1.5–2 mm (or 6–8 point). For printing at full page width with this size of type, a layout with 80–100 nucleotides per line is appropriate (or 60–70 if there are spaces between the codons). For a single-column layout, 50–60 nucleotides per line is about right. The spacing between the lines of sequence should be as close as is consistent with clarity. Note that sequence data must be submitted to GenBank, EMBL or DDBJ.
JMM does not publish figures whose principal function is to present primary sequence data, since the data can be accessed through the databases. To merit publication, sequence figures must be justified by the additional annotation they present; they should normally be limited to regions of particular interest. Limited sequence alignments of nucleic acids and proteins are acceptable provided they make a significant point. See above for guidance on presentation of sequence figures. Sequence data that are not suitable for print publication can, where appropriate, be published as online-only supplementary data.
Photographs (halftones). Authors are advised to supply halftones intended for publication as TIFF or EPS files. The resolution should be at least 300 d.p.i. at final size (approx. 1000 pixels wide for a single-column figure; approx. 2000 pixels wide for a double-column figure). For photomicrographs, the scale should be shown by a scale bar.

2.5.12 Colour figures

These are published at no cost to the author, if the Editors believe that colour is essential to show the results. Colour figures should preferably be supplied as TIFF or EPS files. The resolution should be at least 300 d.p.i. at final size

(approx. 1000 pixels wide for a single-column figure; approx. 2000 pixels wide for a double-column figure). The files should preferably be generated as CMYK (4-colour) images, not RGB, as these reproduce better in print.

2.5.13 Supplementary material

Material associated with a paper but not suitable for print publication (e.g. large datasets, sequence alignments, 3D structures or movie files) can be included as online-only supplementary data. Data that are essential for interpretation of the results of the main paper should be included in the main paper. All supplementary data files will be reviewed along with the main paper; these will not be published unless they significantly enhance the paper. The Editors may suggest that figures or tables included within a paper should be converted into supplementary data.

Supplementary data files must not include methods for results that are included in the main paper, nor should they introduce different results or new discussion points.

Submission

- Supplementary data should be uploaded at the time of submission; please indicate the contents of the file in the 'File label' field.
- Supply all supplementary material in the file formats given below.
- Very large files or those requiring specialist software are not suitable as they will be difficult for the reader to download or view.

Presentation

- Supplementary figures and tables should be named Fig. S1, Table S1, etc., and must be cited accordingly in the main paper.
- Provide a heading and, if appropriate, a short legend or text description with each supplementary data item.
- In each file, please include the following information: article title, author names, journal name, affiliation and email address of the corresponding author.

File types

- Material should be submitted in PDF format; .doc(x) or .ppt(x) files are not suitable.
- Multiple figures, tables or text items should be supplied as a single PDF.
- Large datasets can be supplied in Excel format if you wish readers to be able to manipulate the data. If not, please convert to PDF.
- Audio, video and animations can be supplied as .mov, .avi or .mpeg files.
- The preferred file type is PDF; however, please check the full list for other acceptable file types.

Processing of supplementary files

- Supplementary data files will be uploaded alongside the main paper exactly as supplied by the author. It is the authors' responsibility to ensure that all files are presented clearly.

3. Style, Nomenclature and Units – Contents

3.1 Nomenclature of micro-organisms

The correct name of the organism, conforming with international rules of nomenclature, must be used; if desired, synonyms may be added in parentheses when the name is first mentioned. Names of bacteria must conform with the current Bacteriological Code and the opinions issued by the International Committee on Systematics of Prokaryotes. See the *International Journal of Systematic and Evolutionary Microbiology* Information for Authors for more details. Names of algae and fungi must conform with the current International Code of Botanical Nomenclature. Names of protozoa must conform with the current International Code of Zoological Nomenclature.

The following may be useful:

- List of Prokaryotic Names with Standing in Nomenclature
- Bergey's Manual of Systematic Bacteriology

3.1.1 Vernacular names

Generic names are singular Latin nouns and do not take a plural verb. Authors should avoid the use of a generic name alone when the reference is to the members of the genus. Thus, 'The strains (species or cultures) of *Salmonella* are…' not 'The *Salmonella*are…'. The latter implies more than one generic name *Salmonella*.

Many micro-organisms are known by their vernacular (common) names as well as by their scientific names. The vernacular name for an organism may vary from language to language or from place to place, even within the same country. There are no rules governing the use of vernacular names.

It is often convenient to use vernacular names coined from the generic names. In these forms, the initial capital letters are dropped and italics are not used. For plural forms of vernacular names, Latin or other plural endings are used, depending primarily on euphony. Thus, the vernacular singular for a member of the genus *Spirillum* is spirillum, and the plural generally used in the English language is spirilla (Latin plural), not spirillums (English plural). Occasionally, more than one common name arises from a generic name, such as treponema (plural treponemata or treponemas) and treponeme (plural treponemes) from*Treponema*.

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3.2 Chemical and biochemical nomenclature

Authors should follow the recommendations of IUPAC for chemical nomenclature, and those of the Nomenclature Committee of IUBMB and the IUPAC–IUBMB Joint Commission on Biochemical Nomenclature for biochemical nomenclature (seehttp://www.chem.qmul.ac.uk/iupac/jcbn).

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3.3 Enzyme nomenclature

The system published in Enzyme Nomenclature (http://www.chem.qmul.ac.uk/iubmb/enzyme) should be followed. Enzyme Commission numbers should be given where appropriate.

For restriction enzymes, use e.g. *Eco*RI **not** EcoRI, etc.; *Hin*dIII **not** *HindIII*, HindIII, *Hind III*, etc.

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3.4 Genetic nomenclature

For bacterial gene names, use e.g. *gyrA* **not** *gyr*A; *arg-1* **not** *arg1* or *arg*1, etc.

The following proposals should be adhered to wherever possible.

Bacteria: Demerec, M. *et al.* (1966) *Genetics* **54**, 61–76 [also *J Gen Microbiol* (1968), **50**, 1–14].

Plasmids: Novick, R. P. *et al.* (1976) *Bacteriol Rev* **40**, 168–189.

Saccharomyces cerevisiae: Sherman, F. (1981) In *The Molecular Biology of the Yeast Saccharomyces*. I. Life Cycle and Inheritance, pp. 639–640 (edited by J. N. Strathern *et al.* New York: Cold Spring Harbor Laboratory).

Aspergillus nidulans: Clutterbuck, A. J. (1973) *Genet Res* **21**, 291–296.

Neurospora crassa: *Neurospora Newsl* (1978), **25**, 29.

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3.5 Abbreviations

Abbreviations must be listed on title page, and defined at first mention in both Summary and main text.

The following need **not** be defined:

aa; ACES; ADA; ADP, cAMP, ATP, etc.; AIDS; BES; Bicine; bp; BSA; CAPS; c.f.u.; CHAPS; CHES; CIE; CM-cellulose; CoA; c.p.m.; Da; DEAE-cellulose; DIG; DMSO; DNA, cDNA, CCC DNA, dsDNA, ssDNA, DNase; DNP; d.p.m., d.p.s.; DTT; ED_{50}; EDTA, EGTA; ELISA; EMS; e.o.p.; EPR or ESR; FITC; FPLC; GC or GLC; GSH, GSSG; HEPES; HEPPS; HPLC; i.d.; IEF; IgG, IgM, etc.; IPTG; IR; kb, kbp; LD_{50}; LPS; LSU; mAb; MES; MIC; m.o.i.; MOPS; MS; NAD, NADP; NMR; nt; NTG; ONPG; ORF; PAGE; PBS; PCR; PEG; PFGE; p.f.u.; P_i, PP_i; PIPES; PMSF; ppGpp, pppGpp; p.p.m.; p.s.i.; PVDF; Py-GC, Py-MS; RBS; RFLP; RNA, mRNA, rRNA, tRNA, RNase; r.p.m.; RT-PCR; SDS, SDS-PAGE; SSU; TCA; TES; TLC; Tricine; Tris; UPGMA; UV; X-Gal.

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3.6 Units

3.6.1 General points

SI units should be used. If non-SI units are used, the equivalent in SI units should also be given, e.g. 1 p.s.i. (6.9 kPa).

For **compound units** (e.g. micrograms per millilitre), use µg ml^{-1} not µg/ml; use 10 µg ampicillin ml^{-1} not 10 µg ml^{-1}ampicillin.

Give **concentrations** as g l^{-1}, etc., or molarity, M, **not** normality, N. The term '%' should be defined as 'w/v', 'v/v' or 'w/w' if this is necessary to avoid ambiguity.

For **radioactivity**, the preferred unit is becquerels (Bq); if given in curies (Ci), the equivalent in Bq **must** be given (1 Ci = 3.7×10^{10} Bq); radioactivity may also be expressed as d.p.s. (1 d.p.s. = 1 Bq) or as c.p.m.

3.6.2 Molecular mass

M_r (relative molecular mass) should be used rather than "molecular weight". "Molecular mass" should be used if values are quoted in daltons (Da) (e.g. molecular mass 20 kDa). Either M_r or molecular mass may be used, but they should not be mixed in any one paper. In table headings and figure axes, for values >1000 use kDa or $10^{-3}\times M_r$.

3.6.3 Absorbance, optical density and attenuance

The term absorbance, A, should be used for the quantity $\log(I_0/I)$ in UV and visible absorption spectrophotometry of samples in which there is negligible scattering or reflection of light. If scattering is considerable, as in spectrophotometric measurements of microbial biomass, the term optical density, OD (or attenuance, D), should be used; the path length of the cell or cuvette, and the make and model of the spectrophotometer, should be specified, because optical design dramatically influences such measurements. If a sample is diluted prior to measuring optical density, the dilution and the diluent should be stated. Readings obtained with instruments designed for turbid samples, such as nephelometers or Klett meters, should be reported in appropriate units. Whenever A, OD or D is used, the wavelength (in nm) of the incident light must be specified (e.g. A_{280}, OD_{600}).

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3.7 Presentation of nucleotide and amino acid sequences

In the absence of a detailed discussion of specific structural features, the nucleotide sequence or proposed secondary structure should not be presented. Such papers should be accompanied by substantial additional experimentation to characterize the gene(s) and products(s) concerned, and by substantial computer analysis. JMM will not normally publish DNA sequences from double-stranded genomes unless the two strands have been sequenced independently.

JMM will not publish figures whose principal function is to present primary sequence data, since the data can be accessed through the databases. To merit publication, sequence figures must be justified by the additional annotation they present; they should normally be limited to regions of particular interest. Sequence alignments of nucleic acids and proteins may be presented using the supplementary data facility.

When making comparisons between nucleotide or amino acid sequences, it is important to use the correct terminology. 'Homology' has a precise biological meaning of 'having a common evolutionary origin'. When a percentage comparison is made, the terms 'identity' or 'similarity', as appropriate, must be used.
Submitted manuscripts containing new sequence data should include, on the title page, the footnote 'The GenBank[/EMBL/DDBJ] accession number for the [16S rRNA gene/*gyrA*, etc.] sequence of XXXXX is XX00000'.

4. Peer Review and Publication – Contents

4.1 Editorial handling of papers

During the online submission process, authors must select an Editor who would be appropriate to handle their paper. To do this, the relevant Editor should be selected from the drop-down list. You may also exclude up to two Editors who you do not wish to handle your paper in the comments section.
Submitted papers are checked by the editorial staff to ensure that they comply with the journal's requirements. If any problems are noticed, the paper will be returned to the author for amendment before being assigned to an Editor. After the above step, papers are assigned by the editorial staff to an Editor with appropriate expertise, who is responsible for making the decision on acceptability; while every effort will be made to select an Editor suggested during the submission process, the editorial office staff reserve the right to assign the paper to another Editor if the one suggested is unavailable or another Editor is considered significantly more appropriate. Before sending a paper to reviewers, the Editor will pre-screen the paper to check that it fulfils certain basic criteria that would make it potentially suitable for JMM: nature of the study, quantity and quality of data, general conclusions and standard of presentation. If the paper does not fulfil these criteria, it may be rejected at this stage, so that the authors can submit the paper to a more appropriate journal without further delay. Papers that pass the pre-screening stage will normally be sent to at least one independent reviewer, but the Editor may also act as a reviewer him/herself. The use of reviewers suggested by authors is at the discretion of the Editor.
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4.2 Submitting a revised manuscript

If revision of a paper is requested, the revised version should be returned within the time specified. If more time is required, the author should contact the Editorial Office (jmm@sgm.ac.uk) or Editor to discuss a new deadline. If revision is delayed by the author without prior agreement, the revised version will be treated as a new submission. When submitting the revised version of a paper, authors should supply the source files for the text and figures, to expedite the publication of the paper if it is accepted.
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4.3 Source files for publication

Our requirements for files intended for publication are different from those for files that will be converted to PDF by the submission system as part of an initial submission. If you are unsure whether your file formats are suitable, please contact the Editorial Office.

4.3.1 Text

The text (including tables, but without embedded figures) must be supplied as a word-processor file. Word files are preferred; .docx files produced in Word 2007 or 2010 can be used as source files. TeX and LaTeX formats can not be used.

4.3.2 Tables

Tables must **not** be supplied as image files (TIFF, PDF, PowerPoint); files containing tables prepared as images (whether provided separately or pasted into a Word file) will be returned to the author and this may delay publication. Tables should be prepared using your word-processor's table functions, with individual entries in individual table cells. They must **not** be supplied as tab- or space-separated text or as multiple entries separated by line breaks in single table cells. Tables prepared in Excel can be accepted but are not desirable.

4.3.3 Equations

Equations that cannot be represented using the keyboard can be prepared using the Word equation editor (in versions up to Word 2003) or MathType. Word 2007/2010 users should **not** use the default equation editor to prepare equations as it is not compatible with any other current software; equations in Word 2007/2010 should be prepared using the MathType equation editor or the 'legacy' equation editor included as part of Word (i.e. a Microsoft Equation 3.0 object, accessible from 'Insert Object' on the 'Insert' ribbon).

4.3.4 Line figures

Line figures should be produced as vector rather than bitmap (raster) images. Acceptable formats are PDF, EPS, CorelDRAW (.cdr; version 15 or earlier), Adobe Illustrator (.ai), Excel (.xls), Word and PowerPoint. Fonts must be embedded for figures supplied as PDF or EPS. TIFF and other bitmap formats are not recommended for line figures; if their use cannot be avoided, the resolution should be at least 600 d.p.i.
Charts prepared in Microsoft Excel should be supplied in Excel format where possible. If they are copied and pasted into another Microsoft application, use Paste Special and select 'Picture (Enhanced Metafile)'.

4.3.5 Halftone figures (photographs)

The preferred format for halftones (i.e. photographic images) is TIFF, but PDF, EPS and JPG/JPEG are also acceptable. If image files are pasted into Word, PowerPoint, Photoshop, etc., in order to add lettering or other annotation or **to combine line and halftone images**, the original unlabelled halftone images should also be supplied.
A final print resolution of 300 d.p.i. or more is recommended; i.e. an image intended to fit in a single column of the journal should be around 1000 pixels wide and an image intended to fit across two columns should be around 2000 pixels wide. Colour images should use CMYK colour (which can be reproduced in print) rather than RGB (which cannot be reproduced faithfully using four-colour printing) (this setting can be accessed in Adobe Photoshop via Image:Mode:CMYK Color, for example). For some colour images, such as fluorescence micrographs, it may be useful to submit an RGB version of the image to be mounted online as supplementary material.

4.3.6 Scanning images
If images must be scanned, a resolution of 300 d.p.i. is usually sufficient for same-size reproduction of halftone (photographic) images without text, whereas 600 or 1200 d.p.i. should be used for figures containing lines and/or text. The scanned image should be cropped to remove as much white space as possible and supplied in TIFF format.

4.3.7 Supplementary material
Files supplied as supplementary material should follow the appropriate guidelines given above. If supplementary files contain material that cannot be represented in print (sound, video, computer programs, etc.), please consult the Editorial Office for advice on suitable formats.
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4.4 Publication ahead of print
JMM has a Papers in Press feature where accepted manuscripts appear online in an unedited format before they are scheduled to appear in print. Unless the authors inform the Editorial Office staff (jmm@sgm.ac.uk) to the contrary, it will be assumed that they agree to their manuscript being used in this way. Please note that the PDF used for peer review, and the manuscript title, subject category and author details that authors have entered into the online submission site, will be used to generate the Papers in Press record.
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4.5 Proof corrections
Proofs are sent by email as a PDF attachment to the email address supplied for the corresponding author. It is the authors' responsibility to inform the Editorial Office of any changes to this email address.
Only typographical and absolutely essential factual changes may be made. Authors may be charged for the correction of non-typographical errors.
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4.6 Electronic and print reprints
Following publication of the final version of their paper, corresponding authors will receive an email including a link to download the published PDF of their paper. The link can be forwarded and used to download the published PDF up to 25 times.
Reprints of articles published in JMM can be ordered through the SGM Reprints Service. Authors will receive full details of this when their paper has been published. Payment can be made by credit or debit card, Paypal or by purchase order. Orders placed by purchase order will be subject to approval before being passed for printing.
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4.7 Cover illustrations
The Editors welcome the submission of pictures for possible use on the front cover. Pictures need not be linked with a paper in the journal.

ANEXO F. REGRAS PARA SUBMISSÃO: BRAZILIAN JOURNAL OF INFECTIOUS DISEASES

INSTRUCTIONS TO AUTHORS

- Editorial Politic
- Submission of manuscripts
- Checklist for submitted manuscripts

ISSN 1413-8670 ***printed version***
ISSN 1678-4391 ***online version***

Editorial Politic

Instructions for submission

Authors should submit their articles through the website: (www.sgponline.com.br /bjid).

Manuscripts may be submitted within designated categories of communication, including:

• original basic or clinical research;
• presentation and discussion of case;
• short reports of new methods or observations;
• presentations or reviews of state of the art;
• Letters to the editor about the previous publications;
• editorials that contain ideas, suggestions, and comments.

Supplements BJID include articles under a unified theme, as in a summary of presentations from a symposium, or focusing on a specific pathogenic process or antimicrobial agent.

Published

The BJID is published bimonthly (February, April, June, August, October, December) by Elsevier. The paper's editors reserve the right to edit manuscripts to improve clarity, grammar and style. The authors have the opportunity to review these changes before the creation of the galley proofs. The content changes made after the galley proofs are sent for review cost will be borne by the author. The BJID not accept items that overlap or duplicate publications elsewhere.

Submission of manuscripts

Manuscripts submitted to Brazilian Journal of Infectious Diseases should be sent to the editor in chiefhttp://www.sgponline.com.br/bjid/sgp/ address. Each manuscript will be assigned a registration number, and the author will be notified that the manuscript is complete and appropriate to begin the review process.

SGP (Publication Management System) is a system that allows submission of papers and of the editorial board in a fully web. The SGP is based on access levels, so when a user submits a manuscript for review and approval, it remains only to display the author and the editorial board, keeping it safe from other authors who have not participated in the research and development work. It is crucial that the author who submits papers in the SGP has registered an email which he uses, because all the information and notices concerning status, approval or refusal of work will be done by email. If the author changes the email, they can update their cadastral data through the section "Registration" on the navigation bar.

The manuscript submission process is based on nine steps of which the author has been filling the items relating to his work as a title, as described (keywords), abstract, additional information about the possibility of sending images or graphics (in JPG format only) and mount the article content through a graphical interface page with the program similar to MS Word, can be edited to create tables, insert images and apply formatting to your text. In the last step shows a PDF version of your manuscript and choose to approve or delete your submission.

Checklist for submitted manuscripts

1. Please provide a cover letter with your submission specifying the corresponding author as well as an address, telephone and facsimile number.

2. Submit four copies of your manuscript (original plus three copies and a disk of the manuscript - Word Perfect/Word for Windows), each with a complete set of original illustrations.

3. The entire manuscript (including tables and references) must be typed double-spaced and printed on standard-sized paper. The left and right margins must be at least 3cm.

4. The entire manuscript must be typed in a font size of at least 12 points.

5. Please number pages beginning with the title page (title page is

page 1).

6. The order of appearance of material in all manuscripts should be as follows: title page, abstract, text, acknowledgements, references, tables, legends for figures, figures.

7. The title page must include a title of not more than three printed lines (250 letters and spaces), authors (no titles or degrees), institutional affiliations, a running headline of not more than 40 letters and spaces, a name and complete address to which correspondence and reprint requests should be sent, and footnotes indicating sources of financial support and changes of address.

8. Abstract (maximum 250 words) must be on a separate page before the introduction. Do not submit an abstract with correspondence.

9. Acknowledgements of persons who assisted the authors should be included on the page preceding the references.

10. References must begin on a separate page.

11. References must be cited on (not above) the line of text and in brackets instead of parentheses, e.g., [7,8].

12. References must be numbered in the order in which they appear in the text. References not cited in the text cannot appear in the reference section. References only or first cited in a table or figure are numbered according to where the table or figure is cited in the text. For instance, if a table is placed after reference 8, a new reference cited in table 1 would be reference 9.

13. Reference citations must follow the format established by the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (see examples in Instructions to Authors).

14. If you reference your own unpublished work (i.e., an "in press" article) in the manuscript that you are submitting, you must enclose three copies of the "in press" article and an acceptance letter from the journal.

15. If you cite unpublished data that are not your own, you must provide a letter of permission from the author of that publication.

16. Please provide four glossy or laser-produced prints of each figure that you are submitting. Label all figures clearly with first author's name and figure number (place typed label on back of figure).

17. Provide a figure legend for each figure. Figure legends must be on a separate page at end of manuscript.

Please refer to Instructions to Authors for further information regarding the title page, abstract, references,

tables and figures, and style.

The articles could be sent by e-mail or through the web site www.bjid.com.br.

ANEXO G. ARTIGO PUBLICADO EM REVISTA INTERNACIONAL

Diagnostic Microbiology and Infectious Disease 79 (2014) 401–404

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Bacteriology

Is prediffusion test an alternative to improve accuracy in screening hVISA strains and to detect susceptibility to glycopeptides/lipopeptides?

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Staphylococcus aureus

ABSTRACT

The characterization of heteroresistant vancomycin-intermediate *Staphylococcus aureus* strains (hVISA) is even more challenging, as no routine standardized laboratory methods are available. A total of 124 *S. aureus* isolates recovered from inpatients attended in hospitals of Santa Catarina State, Southern Brazil, were evaluated. The MIC of vancomycin, teicoplanin, and daptomycin was determined by Etest and prediffusion tests using NeoSensitabs® tablets. All isolates were susceptible to vancomycin (MICs: 0.5–3 μg/mL) by Etest. However, according to prediffusion test, 17 isolates presented reduced susceptibility to vancomycin, and of these, 12 were confirmed as hVISA using populational analysis. Considering daptomycin, prediffusion results were in agreement with susceptibility data (MICs), as all isolates were susceptible. Considering that characterizing hVISA is challenging and that MIC determination is not adequate to characterize this phenotype, prediffusion test was a viable alternative to screening hVISA and reduced susceptibility to vancomycin. It was simple and low cost, with accuracy comparable to other well-established methods.

1. Introduction

Vancomycin is a glycopeptide known since 1955, when it was placed at the disposal of medicine through the work of McCormick (McCormick et al., 1955). Initially, due to the success of methicillin, oxacillin, and other isoxazolepenicillins, it was not often used, though with the emergence of methicillin resistance in the 1960's, it came to be used quite often. In 1996, a strain of *Staphylococcus aureus* intermediate to vancomycin (vancomycin-resistant *S. aureus* [VISA]) was identified in Japan (Hiramatsu, 1997). This phenotype did not have a wide dissemination, and the drug most frequently used to treat infections caused by methicillin-resistant *S. aureus* (MRSA) is still vancomycin, as well as the daptomycin and linezolid (Jones, 2006; Van Hal and Fowler, 2013).

Detection of in vitro susceptibility of isolates to these drugs is challenging. Since 2009, the CLSI (2013) no longer recommends disk diffusion to determine vancomycin susceptibility due to its high molecular weight resulting in decreased diffusion in culture media (Heather et al., 2010). Based on current CLSI recommendations, susceptibility to linezolid and teicoplanin may be determined by disk diffusion method, while vancomycin and daptomycin susceptibilities should be assessed by dilution methods or some specific diffusion tests in agar. These tests are extremely laborious and expensive, making them difficult to implement, especially in developing countries.

Taking into account the seriousness of MRSA infections, early and aggressive antimicrobial therapy is an important part to reduce mortality. Treatment failures in infections caused by susceptible isolates are primarily due to heteroresistant vancomycin-intermediate *S. aureus* strains (hVISA), a subpopulation with reduced susceptibility to vancomycin. Thus, in vitro susceptibility may not be enough for the physician to achieve treatment success. The evaluation of tolerance to glycopeptide antibiotics is important for predicting treatment failure (Van Hal and Paterson, 2011) and can be considered the first step to preventing and controlling the emergence of vancomycin resistance in *S. aureus* (Howden et al., 2010). As the hVISA resistance phenotype manifests itself heterogeneously and is a minor component of the bacterial population (1 in 10^6 microorganisms), the methods commonly used in clinical microbiology laboratories (MIC determination by Etest or microdilution) fail to detect potential resistance, and vancomycin therapy may fail (Szabó et al., 2011).

NeoSensitabs® (Rosco Diagnostica, Taastrup, Denmark) are tablets containing antimicrobial (9 mm diameter and 1.5 mm thick) stable at room temperature, which were developed to evaluate bacterial

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susceptibility to high-molecular-weight drugs such as vancomycin, teicoplanin, daptomycin, and colistin. The initial prediffusion allows a homogeneous diffusion of the antibiotic in the culture medium, making possible to discriminate strains with reduced susceptibility to these agents. The aim of this study was to evaluate the prediffusion methodology for the detection of decreased susceptibility to glycopeptides and daptomycin as well as its use in the detection of hVISA isolates.

2. Methods

2.1. Samples

Clinical isolates (n = 124) were collected (March 2009 to February 2013) from inpatients in three hospitals in Florianópolis (Hospital de Caridade, Hospital Governador Celso Ramos, and Cardio SOS) and a hospital in Blumenau (Hospital Santa Isabel), all located in Santa Catarina state, Southern Brazil. One isolate per patient was considered. All isolates were used, and there was no selection bias. Identification was done using the following testes: Gram staining, catalase production, mannitol fermentation, coagulase, and DNase production. Susceptibility to methicillin was determined by cefoxitin disk diffusion, according to the interpretive criteria of CLSI (M100-S23).

2.2. Phenotypic tests for screening and confirmation of hVISA

Three screening tests were used for the detection of hVISA strains. BHI agar plates containing 4 μg/mL of vancomycin and 16 g/L of pancreatic digest of casein were inoculated with a prepared 0.5 McFarland standard inoculum. After 24 h of incubation at 35–37 °C, the growth of more than 20 colonies was considered a positive test for hVISA (Satola et al., 2011). Using Etest® Glycopeptide Resistance Detection (GRD) (bioMerieux, Durham, NC, USA), the isolate was considered hVISA when the MIC for teicoplanin is 12 μg/mL or 8 μg/mL for teicoplanin and vancomycin after 48 h (Van Hal et al., 2011). The Etest® macromethod (bioMerieux) was performed using a 2.0 McFarland inoculum on BHI agar plates and readings taken at 24 and 48 h, where an MIC of 8 μg/mL to vancomycin identified hVISA isolates (Van Hal et al., 2011).

All isolates with at least 1 positive screening test were confirmed through population analysis profile/area under the curve (PAP-AUC). The area under the curve (AUC) was calculated using Mu3 (hVISA) as a control strain. For confirmation of an hVISA isolate, the ratio of AUC for the isolate divided by that of Mu3 should be greater than or equal to 0.9 and non-hVISA isolates had a PAP-AUC <0.9 (Wootton et al., 2001).

2.3. Minimum inhibitory concentration (MIC)

MIC values to vancomycin, teicoplanin, and daptomycin were obtained by the Etest® methodology (bioMerieux). Interpretation was performed, following CLSI (M100-S23) guideline.

2.4. Prediffusion tests

Neosensitabs® containing 30 μg vancomycin, 30 μg teicoplanin, or 30 μg daptomycin/100 μg calcium (Rosco Diagnostica) and prediffusion tests were performed following the manufacturer's guidelines (Supplement User's Guide, 2010). The tablets were placed on the surface of Mueller Hinton agar (bioMerieux). They were inverted and incubated for 2 h at room temperature. After this period, plates were incubated for further 18–22 h at room temperature to ensure the complete diffusion of antibiotics. Then, plates were inoculated with bacteria and incubated at 35 °C for 24 h and inhibition evaluated. Isolates with vancomycin ≤22 mm and/or teicoplanin <20 mm inhibition zones were considered VISA/hVISA. For daptomycin, isolates <2 mm were defined as resistant.

2.5. Quality control

To ensure the quality and accuracy of the test results, *S. aureus* strains ATCC 29213 (MSSA), ATCC 43300 (MRSA), ATCC 700698 (hVISA), and ATCC 700699 (VISA) were used.

3. Results

All isolates were susceptible to vancomycin, considering E-test results. MICs were 0.5 μg/mL (0.8%), 0.75 μg/mL (0.8%), 1.0 μg/mL (17.7%), 1.5 μg/mL (42.7%), 2.0 μg/mL (32.3%), and 3.0 μg/mL (5.6%) (Fig. 1). The isolates that had an MIC of 3 μg/mL were considered susceptible to vancomycin since all had MIC obtained by broth macrodilution values ≤2 μg/mL. Considering these data, all isolates would be characterized according to CLSI criteria as susceptible to vancomycin. Seventeen isolates had discrepant results for MIC and prediffusion: they were considered susceptible by the CLSI breakpoints. Among the 17 discrepant isolates, 2 had an MIC of 1.5 μg/mL, 10 presented MIC of 2.0 μg/mL, and 5 MIC of 3.0 μg/mL.

Teicoplanin data indicated MICs of 0.19 μg/mL (0.8%), 0.25 μg/mL (0.8%), 0.38 μg/mL (1.6%), 0.5 μg/mL (3.2), 0.75 μg/mL (1.6%), 1.0 μg/mL (4.8%), 1.5 μg/mL (17.7%), 2.0 μg/mL (16.9%), 3.0 μg/mL (33.9%), 4.0 μg/mL (13.7%), 6.0 μg/mL (1.6%), 8.0 μg/mL (2.4%), and 12.0 μg/mL (0.8%) (Fig. 2). Discrepancies were observed for 14 isolates, which were classified as intermediate according to prediffusion testing but considered susceptible according to CLSI criteria. Among them, 1 had an MIC of 1.5 μg/mL, 7 at 3.0 μg/mL, 4 at 4.0 μg/mL, and 2 at 8.0 μg/mL.

Vancomycin and teicoplanin susceptibility data demonstrated that 21 clinical isolates were intermediately susceptible to vancomycin and/or teicoplanin, which indicated these isolates were VISA using the prediffusion method.

All isolates were submitted to screening tests for the hVISA phenotype (GRD, Etest® macromethod, and agar screening with vancomycin), and of those that tested positive for any of the 3 screening tests, the phenotype was confirmed by PAP-AUC. Of the 124 isolates, 12 (9.7%) were characterized as hVISA (Table 1).

Based on the above data, 91.7% sensitivity, 83.1% specificity, a positive predictive value of 52.4%, a negative predictive value of 97.1%, and an accuracy of 89.5% were established for prediffusion test (Table 2).

Daptomycin data indicated MIC values of 0.125 μg/mL (3.2%), 0.19 μg/mL (5.6%), 0.25 μg/mL (8.9%), 0.38 μg/mL (23.4%), 0.5 μg/mL (33.9%), 0.75 μg/mL (21%), and 1.0 μg/mL (4%) (Fig. 3). All isolates presented, therefore, a susceptible phenotype.

Prediffusion results for ATCC700698 (hVISA) and ATCC 700699 (VISA) are demonstrated in Fig. 4, as well as the results of 2 clinical isolates (SI11 and L10).

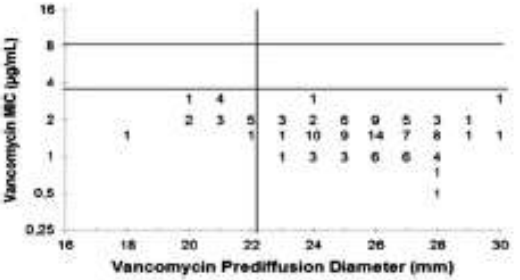

Fig. 1. Scattergram with distribution of vancomycin prediffusion results compared to MICs. Horizontal lines represent the CLSI breakpoints for susceptibility (≤4 μg/mL) and resistance (>8 μg/mL); vertical line represents the breakpoint for a prediffusion-positive result (susceptible ≤22 mm).

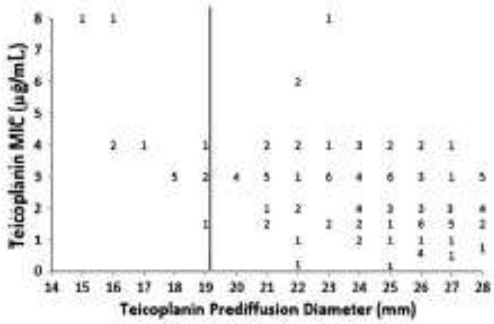

Fig. 2. Scattergram with the distribution of teicoplanin prediffusion results compared to MICs. Vertical line represents the breakpoint for a prediffusion-positive result (susceptible >18 mm).

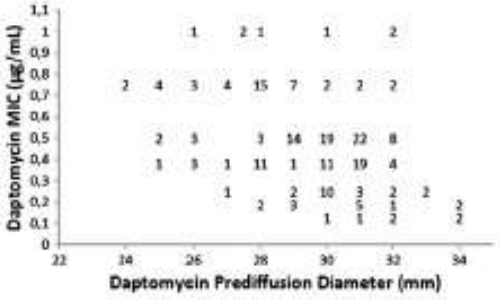

Fig. 3. Scattergram with the distribution of daptomycin prediffusion results compared to MICs. All isolates were susceptible.

Table 1
Comparison of PAP results (hVISA and non-hVISA) with prediffusion results.

	hVISA	Non-hVISA	Total
Positive prediffusion	11	10	21
Negative prediffusion	1	102	103
Total	12	112	124

4. Discussion

The prediffusion method is an alternative to conventional methods, allowing antimicrobials with high molecular weight to be evaluated by this alternative disk diffusion, since by conventional methodology they do not have cutoffs in CLSI guidelines.

Surprisingly, few studies have been published using prediffusion for the assessment of *S. aureus* susceptibility to glycopeptides and daptomycin (Katz et al., 2008; Nielsen and Casals, 2005). The major disadvantage of prediffusion considering glycopeptide susceptibility is that the methodology is unable to differentiate between hVISA and VISA isolates. Besides, it is a qualitative methodology without the possibility to determine MICs. Its qualitative data have presented a good correlation with MIC results, suggesting this test may be used as an alternative test.

In this study, prediffusion data for daptomycin were also highly consistent with MIC results, which were also observed by other authors (Katz et al., 2008). Altogether, these data may support the clinical use of prediffusion test. However, more data must be generated to confirm this hypothesis.

Since 2009, CLSI no longer recommends disk diffusion methodology for vancomycin, requiring clinical laboratories to determine the MIC. Despite being a microdilution reference test, MIC testing is somehow laborious and requires validation. Therefore, several laboratories use the Etest® as an alternative methodology to determine MIC, which is more expensive and tends to overestimate the MIC (Van Hal et al., 2011). However, it is practical and shows a strong correlation with clinical results, justifying its wide usage.

A meta-analysis published in 2012 correlated vancomycin MIC values determined by Etest® with therapeutic failure. The study found a significant correlation between isolates having an MIC ≥ 1.5 µg/mL and therapeutic failure, with an odds ratio of 1.74 (95% confidence interval: 1.34–2.21; $P < 0.01$) (Van Hal et al., 2012). Thus, we used the MICs determined by Etest, in an attempt to find a correlation between the results of prediffusion and Etest.

Several phenotypic tests have been used to detect hVISA as the heteroresistant phenotype has no reliable molecular characterization marker(s) and required the use of phenotypic tests with varying sensitivities and specificities. Satola et al. (2011) reported that screening on BHI agar with 4 µg/mL of vancomycin presents 91% sensitivity and 94% specificity rates, while the Etest® macromethod shows a sensitivity of 57% and specificity of 96% and Etest ® GRD shows a sensitivity of 57% and specificity of 97%.

This study has some limitations. First, the methodology used to determine the MIC was the Etest®, even though the gold standard is the broth microdilution. Second, all isolates tested were susceptible to daptomycin, making it necessary to evaluate the performance of the prediffusion-resistant isolates to determine if the method may present good accuracy for this antimicrobial agent.

These data indicated that when comparing the prediffusion method used for detecting hVISA, the specificity (83.1%) and sensitivity (91.7%) rates from this study were similar to other widely used tests. The results demonstrated that, despite being infrequently used, this test could be viable and effective in screening clinical isolates for an hVISA phenotype. The high negative predictive value (97.1%) allows for testing negative, excluding the possibility of that

Table 2
Results showed that a disagreement between the prediffusion and screening tests for hVISA.

Isolate	Vancomycin (mm)	Teicoplanin (mm)	Etest GRD vancomycin/teicoplanin (µg/mL)	Agar screening	Etest macromethod (µg/mL)	PAP-AUC	Interpretation
S01	21	21	1.5/4	No	8	0.8	False positive
S05	22	18	2/6	No	8	0.77	False positive
S09	22	19	1/1.5	No	6	0.72	False positive
S010	21	17	1.5/6	No	8	0.86	False positive
S012	21	18	2/8	Yes	12	0.89	False positive
S026	22	18	1.5/6	Yes	6	0.78	False positive
S028	21	19	3/8	Yes	8	0.84	False positive
L4	25	18	1.5/2	Yes	4	0.83	False positive
L10	23	20	2/8	Yes	4	0.82	False negative
L64	21	20	1.5/4	No	8	0.79	False positive
L80	24	19	1/4	No	3	0.71	False positive

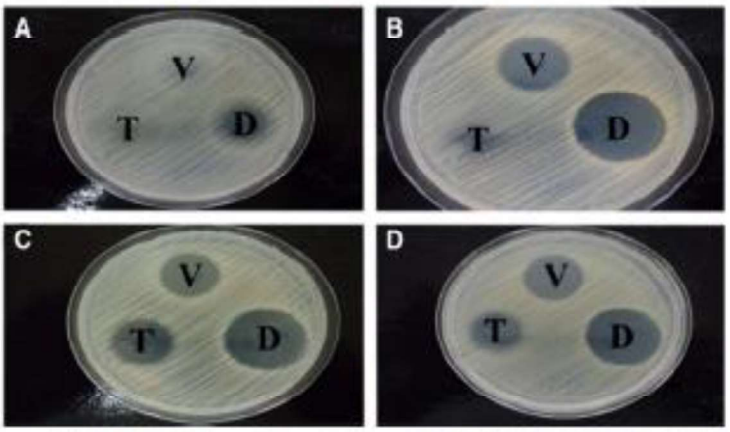

Fig. 4. The picture above illustrate prediffusion, highlighting the control strains: (A) Mu50 ATCC 700699 (VISA), (B) Mu3 ATCC 700698 (hVISA), and 2 clinical isolates, (C) SI11 and (D) L10, both having the hVISA phenotype. V = vancomycin; T = teicoplanin; D = daptomycin.

the phenotype. Taking into account that all positive screening tests should be confirmed with PAP-AUC, the prediffusion method, as a simple and low-cost test, should be considered not only as a useful test to assess the susceptibility of *S. aureus* to glycopeptides and daptomycin, but also as a screening test for hVISA.

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ANEXO H. ARTIGOS PUBLICADOS EM REVISTAS NACIONAIS

J Bras Patol Med Lab • v. 47 • n. 2 • p. 151-156 • abril 2011

ARTIGO DE REVISÃO
REVIEW ARTICLE

Staphylococcus lugdunensis: um olhar diferenciado no laboratório clínico

Primeira submissão em 28/12/10
Última submissão em 23/02/11
Aceito para publicação em 28/02/11
Publicado em 30/04/11

Staphylococcus lugdunensis: *a different view in the clinical laboratory*

Alessandro Conrado de Oliveira Silveira[1]; Pedro Alves d'Azevedo[2]

unitermos

Staphylococcus lugdunensis

Estafilococos coagulase negativos

Endocardites

Clumping factor

Cefoxitina

resumo

Os estafilococos coagulase negativos (ECNs) são cocos Gram-positivos usualmente considerados contaminantes em laboratórios de microbiologia clínica. Apesar de pertencer a este grupo, *Staphylococcus lugdunensis* pode causar infecções complicadas, como endocardites, infecções de pele e tecidos moles, osteomielites, entre outras. Além da formação de biofilmes, apresenta patogenicidade similar ao *Staphylococcus aureus*. É um dos principais agentes causadores de endocardites, com taxa de mortalidade de até 70%. Pode ser confundido com *S. aureus* quando se utilizam testes rápidos para sua identificação, como a pesquisa de *clumping factor*, no caso do teste de coagulase em lâmina, ou em testes de aglutinação direta em látex. Pode ser facilmente identificado por meio de provas bioquímicas acessíveis, como a presença de atividade da ornitina descarboxilase e pirrolidonil arilamidase (PYR). Apresenta sensibilidade à maioria dos agentes antimicrobianos, devendo ser pesquisada rotineiramente a presença de betalactamases e do gene *mecA* por meio de testes com cefalosporina cromogênica e suscetibilidade à cefoxitina, respectivamente. Convém salientar que os critérios interpretativos utilizados para avaliar a sensibilidade à cefoxitina são os mesmos preconizados para *S. aureus* e diferentes dos utilizados para os outros ECNs. Apesar de incomum, o *S. lugdunensis* é um patógeno com acentuada virulência que deve ser constantemente identificado, pois raramente poderá ser considerado contaminante quando isolado de sítios estéreis.

abstract

Coagulase-negatives staphylococci (CNS) are Gram-positives cocci commonly regarded as contaminants in clinical microbiology laboratories. Despite belonging to this group, Staphylococcus lugdunensis may cause complicated infections such as endocarditis, skin infections and soft tissue, osteomyelitis, among others. Apart from the formation of biofilms, it has pathogenic features similar to Staphylococcus aureus. It may be mistakenly identified as S. aureus when using rapid identification tests, such as clumping factor in slide coagulase or in agglutination latex tests. It is easily identified through available biochemical tests, such as the presence of ornithine decarboxylase and pyrrolidonyl arylamidase (PYR). It presents sensitivity to most antimicrobial agents. Furthermore, the presence of beta-lactamase and mecA gene should be routinely investigated by testing with chromogenic cephalosporin and cefoxitin susceptibility, respectively. It is convenient to highlight that the interpretative criteria used to evaluate cefoxitin sensitivity are the same recommended for S. aureus and different from those used for other CNS. Despite the fact it is atypical, S. lugdunensis is a virulent pathogen, which must be accurately identified insofar as it will rarely be deemed as a contaminant when isolated from sterile sites.

key words

Staphylococcus lugdunensis

Coagulase-negative staphylococci

Endocarditis

Clumping factor

Cefoxitin

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Introdução

Os estafilococos coagulase negativos (ECNs) são um grupo numeroso e heterogêneo de cocos Gram-positivos, geralmente associados à contaminação de amostras clínicas. Apresentam baixa patogenicidade, necessitando de uma porta de entrada para causar infecções[7, 8, 28, 30].

Entre os ECNs, o *Staphylococcus lugdunensis* surge como patógeno de características ímpares, pois, apesar de pertencer a este grupo, demonstra patogenicidade semelhante à do *Staphylococcus aureus*, apresentando elevadas morbidade e mortalidade associadas[9, 11, 20].

Atualmente, a prevalência de infecções por *S. lugdunensis* é baixa, variando de 0,6% a 13% entre todos os ECNs isolados em laboratórios de microbiologia clínica. Sua incidência tem aumentado nos últimos anos devido à correta identificação microbiológica[12, 14].

Causa essencialmente infecções em pacientes imunocomprometidos e está associado a extensa gama de processos infecciosos, principalmente infecções de pele e tecidos moles, mas também infecções invasivas, como endocardites, artrite, osteomielite, discite, peritonite, entre outras[9, 12, 13, 28, 31].

A identificação bioquímica da bactéria apresenta algumas dificuldades. A presença de proteínas de superfície específica pode levar às provas de coagulase em lâmina e pesquisa de *clumping factor* positivas, sugerindo *S. aureus*. Provas bioquímicas adicionais para confirmação são necessárias, como o teste pirrolidonil arilamidase (PYR) e ornitina descarboxilase[17].

O objetivo dessa revisão é avaliar a identificação de *S. lugdunensis*, destacar a importância de seu isolamento, descrever sua patogenicidade e discutir os testes de susceptibilidade aos antimicrobianos.

Características

O *S. lugdunensis* foi descrito em 1988 por Freney *et al*[11]. Foi isolado em Lyon, França, explicando a origem do nome, pois *lugdunum* significa Lyon em latim. É um pequeno coco Gram-positivo (0,8-1 µm de diâmetro), catalase-positivo, podendo ocorrer isolado, aos pares e em cadeias. É um anaeróbio facultativo, podendo crescer em temperaturas de 30°C a 45°C e em concentrações de cloreto de sódio de até 15%[11].

Isolados podem apresentar variações na pigmentação e morfologia colonial. As colônias variam de 1 a 4 mm de diâmetro, podendo modificar a coloração para amarelo ou dourado após três a cinco dias de incubação ou permanecer sem pigmentação. A variação na morfologia e pigmentação colonial ocorre devido às pequenas colônias variantes, subpopulações que apresentam crescimento lento, sugerindo a presença de microbiota mista, dificultando a interpretação das culturas[19].

Raramente encontrado na cavidade nasal e isolado mais frequentemente na região perineal, faz parte da microbiota da pele. Em estudo realizado utilizando culturas inguinais de 140 pacientes, houve crescimento de *S. lugdunensis* em 23% das mulheres e 19% dos homens[40].

Discussão

Patogenicidade

Como outros ECNs, a habilidade de aderir e formar biofilmes em superfícies estranhas é a principal responsável pela patogenicidade de *S. lugdunensis*. Os biofilmes são estruturas multicelulares formadas por bactérias fortemente aderidas à superfície e cobertas por uma matriz extracelular polimérica. Promovem proteção contra a resposta imune do hospedeiro, bem como resistência às doses dos antimicrobianos utilizados na terapêutica[10].

O *S. lugdunensis* se liga a várias proteínas solúveis, como colágeno, imunoglobulina G, fibronectina, plasminogênio, entre outras. Além disso, possui uma proteína que pode se ligar ao fator de von Willebrand. As plaquetas e células endoteliais produzem o fator de von Willebrand, glicoproteína responsável pela ligação de colágeno e plaquetas em locais de injúria vascular[21].

Estudos fornecem evidências que demonstram possuir proteínas de superfície associadas que permitem a adesão bacteriana às células do hospedeiro bem como às superfícies estranhas, um passo crítico na formação de biofilmes. A habilidade de se ligar ao fibrinogênio e ao fator de von Willebrand pode justificar a prevalência do microrganismo em endocardites[12].

O fibrinogênio é a maior proteína presente no plasma sanguíneo, atuando no controle da perda de sangue em tecidos danificados por meio da formação de coágulos de fibrina. Também atua na resposta às infecções, visto que a clivagem enzimática de fibrinogênio em fibrina produz fibrinopeptídeos, que são imunoestimuladores[41].

O *S. lugdunensis*, assim como o *S. aureus*, apresenta uma proteína de superfície chamada proteína ligadora de

fibrinogênio, conhecida também como *clumping factor*. É codificada por um gene chamado Fbl (*S. lugdunensis*) e ClfA (*S. aureus*). É um importante fator de virulência, pois se liga no terminal da cadeia γ do fibrinogênio, no mesmo local da integrina plaquetária. As proteínas Fbl e ClfA têm elevada similaridade (62%), apesar de a proteína do *S. aureus* apresentar uma afinidade 10 vezes maior pelo fibrinogênio. Tal fato pode explicar por que alguns isolados de *S. lugdunensis* exibem prova rápida para pesquisa de *clumping factor* negativa. Os anticorpos gerados contra as proteínas Fbl e ClfA podem reconhecer as proteínas heterólogas, sugerindo que apresentem epítopos antigênicos comuns, podendo oferecer proteção cruzada contra *S. aureus* e *S. lugdunensis*[24].

O *S. lugdunensis* produz uma enzima chamada tanase (tanino acil-hidrolase), capaz de hidrolisar taninos. O gene que codifica para esta proteína é o tanA. Ainda não foram encontrados genes similares no GeneBank. Pode-se utilizar reação em cadeia da polimerase (PCR) para detectar tal gene e confirmar rapidamente uma identificação presuntiva de *S. lugdunensis*. Ainda não existem métodos padronizados para detecção direta em amostras clínicas, somente para detecção utilizando a colônia bacteriana[24].

O gene regulador acessório (agr) atua como um regulador global dos fatores de virulência, principalmente exoproteínas secretadas, incluindo enterotoxinas, hemolisinas e numerosas enzimas modificadoras de proteínas do hospedeiro. Análise do *locus* agr de *S. lugdunensis* mostra uma similaridade de 63% com *S. aureus*, explicando a semelhança na patogenicidade entre as duas bactérias[28].

O *S. lugdunensis* não produz α, β, ou γ hemolisina, mas uma δ-hemolisina, termostável, responsável pela atividade hemolítica. Três pequenos peptídeos são responsáveis por esta função: SLUSH A, SLUSH B e SLUSH C, codificados pelo *locus slush* (*S. lugdunensis sinergistyc hemolysin*)[11].

A lisozima é uma enzima fundamental na resposta imune inata a agentes microbianos. O *S. lugdunensis* apresenta uma resistência à lisozima de 400 mg/ml. Outras espécies de ECN são suscetíveis a esta enzima. Em *S. aureus*, a resistência à lisozima é mediada pela presença de uma proteína de membrana (OatA) que acetila o ácido N-acetil murâmico da parede celular, impedindo a ligação da lisozima. O *S. lugdunensis* possui uma OatA homóloga, indicando que o mecanismo de resistência à lisozima é o mesmo descrito para *S. aureus*[12].

Aspectos clínicos

As manifestações clínicas das endocardites causadas por ECN são notadamente diferentes daquelas provocadas por *S. aureus*. Normalmente, o quadro clínico é sutil e não específico e o curso clínico, mais subagudo ou crônico. Raramente representam ameaça de vida ao paciente, especialmente se tratadas precoce e adequadamente. Entretanto, o *S. lugdunensis* é uma exceção devido ao seu caráter agressivo[4, 8, 18, 45].

Ao contrário dos outros ECNs, o *S. lugdunensis* usualmente causa endocardite da válvula nativa, envolvendo principalmente a parte esquerda do coração, frequentemente complicada por uma rápida destruição da válvula (21%) e formação de abscesso paravalvular (23%), muitas vezes exigindo a substituição da válvula. Em 32% dos casos foram verificados embolia sistêmica e focos metastáticos supurativos[38].

Alguns estudos demonstram a presença de até 18% de *S. lugdunensis* entre os ECNs causadores de endocardites, com taxa de mortalidade de 70%[20, 23, 32, 33, 36, 38, 45].

Vários trabalhos demonstram bacteremia e choque séptico induzidos por *S. lugdunensis*. Foi relatado um caso que resultou da transfusão de bolsa de plaquetas contaminada. Também há relato de bacteremia e choque séptico 48 horas após extração dentária[12].

Infecções de pele e tecidos moles representam um proeminente número das infecções causadas por *S. lugdunensis*. Estudo de 63 meses mostrou que 55% dos 155 isolados de *S. lugdunensis* foram provenientes de abscessos, feridas ou celulites[7,40].

O *S. lugdunensis* também é importante causador de infecções ósseas e articulares. Em estudo prospectivo de quatro anos em pacientes submetidos a cirurgias ortopédicas, foi isolado em 3% do total de 212 ECNs[33]. Outro estudo revelou que, durante um período de 40 meses, o *S. lugdunensis* respondeu por 1% dos 601 ECNs isolados de pacientes com infecções ortopédicas, incluindo feridas cirúrgicas e próteses infectadas[21]. Osteomielite foi documentada em algumas ocasiões. O microrganismo foi relacionado com osteítes de crânio, infecções posteriores a cirurgias neurológicas, infecção pós-operatória em cirurgias de joelho, espondilodiscite e osteomielite de vértebra. Trauma e imunossupressão foram fatores de risco[3, 21, 22, 34, 37].

Em estudo com pacientes com e sem infecções orais foi demonstrada a presença de *S. lugdunensis* naqueles com abscessos dentários e osteomielite, porém não foi encontrado em indivíduos saudáveis. Em estudo *in vivo*, os *S. lugdunensis* isolados desses pacientes induziram resposta inflamatória e extensas alterações patológicas, como edema intersticial, infiltração inflamatória celular e destruição

muscular, sugerindo que possa ser o microrganismo associado à doença[42].

Foram relatadas infecções do sistema nervoso central (SNC) relacionadas com o *S. lugdunensis*, incluindo abscessos cerebrais e meningite[14].

Infecções oculares e do trato urinário também foram descritas como causadoras de peritonites[12].

Aspectos laboratoriais

O *S. lugdunensis* produz acetoína, reduz o nitrato, apresenta atividade de ornitina descarboxilase e PYR, oxidase e fosfatase alcalina negativas, é sensível à novobiocina e possui variável resistência à polimixina B[11, 12].

Um sinergismo de ação hemolítica de *S. lugdunensis*, semelhante à atividade das hemolisinas de *S. aureus*, pode ser demonstrado quando são realizadas estrias perpendiculares de β-hemolisina estafilocócica (*Staphylococcus intermedius*) em sangue de carneiro, demonstrada com uma região de completa hemólise na zona de proximidade entre as estrias[11, 12].

É um coagulase-negativo, porém uma variável percentual de isolados apresenta *clumping factor* (coagulase ligada), podendo gerar resultados positivos quando realizado teste rápido de aglutinação em lâmina com plasma ou vários testes comercias de aglutinação em látex[17, 43].

A detecção do *clumping factor* é um teste rotineiramente utilizado para diferenciar *S. aureus* de ECN. Tal teste, realizado sem a coagulase livre (tubo), é a maior causa de erro na identificação de *S. aureus*, visto que o *S. lugdunensis* também pode apresentar *clumping factor*[4, 27].

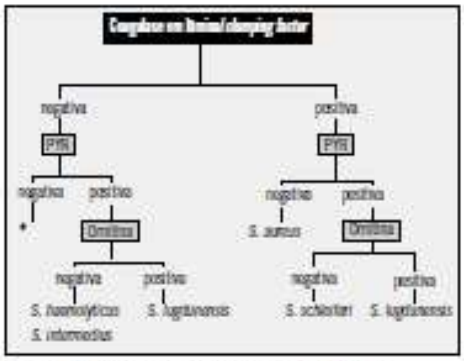

Figura 1 – *Fluxograma de identificação simplificada de S. lugdunensis.*
Adaptado de POUTANEN, S. M.; BARON, E. J. Staphylococcus lugdunensis: a notably distinct coagulase-negative Staphylococcus. Clinical Microbiology Newsletter, v. 23, n. 19, p. 147-50, 2001.
**ECNs outros que S. lugdunensis, S. haemolyticus, S. schleiferi e S. intermedius. PYR: pirrolidonil arilamidase; ECN: estafilococos coagulase negativo.*

Os ECNs não são rotineiramente identificados ao nível de espécie em laboratórios de microbiologia clínica. Normalmente, as culturas com estafilococos são testadas para identificar *S. aureus*, em geral utilizando um teste de aglutinação rápida em lâmina ou pesquisa de proteínas de superfície. Um resultado negativo para o teste em lâmina deve ser seguido de um teste de coagulase em tubo para confirmar tratar-se de um ECN. A intensa virulência associada a *S. lugdunensis* é uma forte razão para a identificação deste microrganismo ao nível de espécie quando há suspeita de infecção, principalmente se isolada de sítios estéreis[11].

O *S. lugdunensis* é facilmente identificável com testes bioquímicos acessíveis e rotineiros, como PYR e atividade da ornitina descarboxilase, como demonstrado na **Figura 1**[28].

Testes de suscetibilidade

O *S. lugdunensis*, ao contrário dos outros ECNs, permanece sensível à maioria das classes de antimicrobianos. Essa tendência de suscetibilidade, incluindo às penicilinas, não se modifica com base na fonte de infecção. Até hoje foram relatados apenas dois casos de *S. lugdunensis* com presença do gene *mecA*[12, 13].

Pode ser realizado o teste de pesquisa de betalactamase, utilizando cefalosporina cromogênica (Nitrocefin). Em caso de não produção de betalactamase, o isolado pode ser considerado sensível a todos os betalactâmicos. Caso o teste seja positivo, o isolado é resistente a penicilina, carbóxi, ureido e aminopenicilinas, devendo-se realizar o teste com o disco de cefoxitina para verificar a suscetibilidade aos outros agentes betalactâmicos. A porcentagem de produção de betalactamases em *S. lugdunensis* varia de 7% a 40%[2, 5, 12].

Desde 2004, o Clinical Laboratory Standards Institute (CLSI) preconizou o uso do disco de cefoxitina em substituição à oxacilina para predizer a presença do gene *mecA*. Tal substituição ocorreu por duas razões. Primeiramente, a cefoxitina apresenta melhores sensibilidade e especificidade, principalmente no caso dos ECNs. Segundo, o disco de cefoxitina é de mais fácil leitura que o de oxacilina, cuja leitura deve ser realizada de maneira bastante minuciosa e com luz transmitida. As zonas de inibição formadas ao redor do disco de cefoxitina são claras e podem ser lidas facilmente com luz refletida[5, 11, 33].

O isolado possuidor do gene *mecA* produz uma proteína alterada de parede chamada proteína ligadora de penicilina (PBP2a), inviabilizando a terapêutica com qualquer agente betalactâmico. Por isso é fundamental a detecção do gene *mecA*, seja por características fenotípicas (resistência à

cefoxitina ou verificação da presença de PBP 2a mediante testes de aglutinação em látex) ou genotípicas (PCR para detecção do gene *mecA*)[2, 3].

De acordo com o documento M100-S20, os pontos de corte para sensibilidade à cefoxitina são, no teste de disco-difusão, halo ≥ 22 mm, e nos testes diluicionais, concentração inibitória mínima ≤4 µg/ml. No caso de resistência à cefoxitina, o isolado deverá ser considerado resistente a todos os beta-lactâmicos (penicilinas, cefalosporinas, penicilinas associadas a inibidores de betalactamases e carbapenêmicos), exceto as novas cefalosporinas (ceftobiprol) com atividade anti-MRSA[5].

Cabe salientar a importância da identificação correta do ECN, pois, caso se trate de *S. lugdunensis*, este apresenta pontos de corte para interpretação do antibiograma iguais aos preconizados para *S. aureus* e diferentes dos utilizados para os outros ECNs[5].

Em isolados resistentes à eritromicina, deve-se também testar a presença do fenótipo MLSi para detecção da resistência induzível à clindamicina, ensaio conhecido como D teste. No antibiograma, a uma distância de 15 a 26 mm do disco de eritromicina de 15 µg, deve-se colocar o disco de clindamicina de 2 µg. Após 16 a 18 horas verifica-se a formação de um achatamento de halo próximo ao disco de clindamicina, conforme demonstra a **Figura 2**. O isolado deverá ser reportado como resistente à clindamicina[5].

Um fenômeno interessante que ocorre com *S. lugdunensis* é a tolerância à vancomicina. Em isolados sensíveis à vancomicina, são refratários em ensaios de atividade bactericida, que podem ser associados a uma razão da concentração bactericida mínima (CBM) e concentração inibitória mínima (CIM) ≥ 32 µg/ml. Noventa e três por cento de *S. lugdunensis* sensíveis à vancomicina (CIM de 0,5 a 2 µg/ml) demonstraram CBM ≥ 128 µg/ml. Um segundo estudo demonstra resultados similares para teicoplanina. Outros estudos tornam-se necessários para determinar a relevância clínica desses achados[7,12].

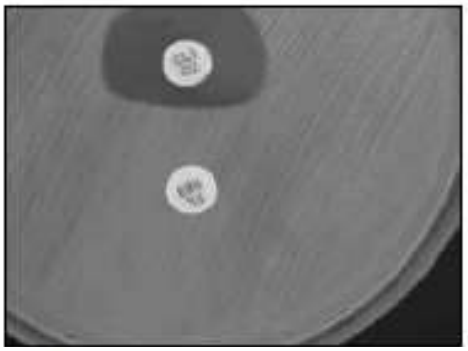

Figura 2 – *D teste: achatamento de halo de clindamicina demonstrando resistência indutível*

Fonte: Cao Roberto Salvino.

Conclusão

O *S. lugdunensis* é um patógeno emergente que tem assumido importância nos últimos 20 anos devido à sua elevada virulência. É fundamental a sua correta identificação, pois raramente poderá ser considerado um contaminante quando isolado de fluidos corporais estéreis.

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EVALUATION OF METHODS IN DETECTING VANCOMYCIN MIC AMONG MRSA ISOLATES AND THE CHANGES IN ACCURACY RELATED TO DIFFERENT MIC VALUES

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SUMMARY

INTRODUCTION: Methicillin-Resistant *Staphylococcus aureus* (MRSA) presenting reduced susceptibility to vancomycin has been associated to therapeutic failure. Some methods used by clinical laboratories may not be sufficiently accurate to detect this phenotype, compromising results and the outcome of the patient. **OBJECTIVES:** To evaluate the performance of methods in the detection of vancomycin MIC values among clinical isolates of MRSA. **MATERIAL AND METHODS:** The Vancomycin Minimal Inhibitory Concentration was determined for 75 MRSA isolates from inpatients of Mãe de Deus Hospital, Porto Alegre, Brazil. The broth microdilution (BM) was used as the gold-standard technique, as well as the following methods: E-test® strips (BioMérieux), M.I.C.E® strips (Oxoid), PROBAC® commercial panel and the automated system MicroScan® (Siemens). Besides, the agar screening test was carried out with 3 µg/mL of vancomycin. **RESULTS:** All isolates presented MIC ≤ 2 µg/mL for BM. E-test® had higher concordance (40%) in terms of global agreement with the gold standard, and there was not statistical difference among E-test® and broth microdilution results. PROBAC® panels presented MICs, in general, lower than the gold-standard panels (58.66% major errors), while M.I.C.E.® MICs were higher (67.99% minor errors). **CONCLUSIONS:** For the population of MRSA in question, E-test® presented the best performance, although with a heterogeneous accuracy, depending on MIC values.

KEYWORDS: Methicillin-Resistant *Staphylococcus aureus*; Vancomycin; Minimal Inhibitory Concentration.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important bacterial pathogens worldwide, especially in healthcare associated infections[7]. As MRSA is almost always multiresistant, vancomycin is the therapy of choice. In 2007, the Clinical and Laboratory Standards Institute (CLSI) determined the reduction of breakpoints for Minimal Inhibitory Concentration (MIC) of vancomycin among *S. aureus* to increase the sensitivity in detecting the non-susceptible isolates[5]. The apparent increase in vancomycin MIC among MRSA, observed in the last years, could represent the first step for the occurrence of fully resistant isolates. Indeed, the emergence of strains has been determined by presenting intermediate resistance (VISA) or hetero-VISA (vancomycin-intermediate *S. aureus*). Besides, increasing proportions of MRSA isolates with high MICs have been observed within the susceptible range, a phenomenon known as vancomycin MIC creep[8,10]. These isolates with MIC creep have been associated with therapeutic failure[13,18], as vancomycin may be ineffective against isolates with MICs between 1 and 2 µg/mL[3].

Several methods with variable sensitivity and specificity are available to determine vancomycin MIC. According to CLSI, broth microdilution (BM) is considered the gold standard[5]. However, because it is time-consuming, a considerable number of clinical laboratories do not use it as routine methodology. Other techniques have been widely used, with variable sensitivity and specificity, such as automated systems, strips with antimicrobial concentration gradient and microdilution commercial panels[6]. The objective of this study was to evaluate the accuracy of several methods in the characterization of vancomycin MIC among clinical MRSA isolates.

MATERIAL AND METHODS

Bacterial isolates: Seventy-five MRSA from Mãe de Deus hospital, a 400-bed general hospital in Porto Alegre, were evaluated in Southern Brazil. Methicillin resistance was first characterized by automated system (MicroScan Walk Away, Siemens®), MRSA phenotype was confirmed by molecular methods (*mec*A gene), described elsewhere[20]. Isolates were maintained (-20 °C) in 10% Skim Milk (Difco, Detroit, USA) with 10% glycerol.

Determination of Minimal Inhibitory Concentration: Vancomycin

Sponsorships: CNPq, FAPERGS.
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MICs were determined by BM[6] and by the following techniques, according to the manufacturer's instructions: E-test® strips (BioMérieux, Marcy l'Étoile, France), M.I.C.E.® strips (Oxoid, Thermo Fisher Scientific, Basingstoke, UK), MicroScan and commercial panels for MIC detection (PROBAC®). Besides, the agar dilution screening test was performed with 3 µg/mL of vancomycin, as proposed by BURNHAM, WEBER & DUNNE[1]. A vancomycin-susceptible strain (ATCC 25923) and a positive control (*Enterococcus faecalis* carrying *vanA* gene) were used for all methodologies.

Statistical analysis: Descriptive statistics were applied, and data were evaluated by ANOVA, followed by the Tukey *post hoc* test. The results were processed using the *Statistical Package for Social Sciences* (SPSS) 17.0. Results statistically significant were considered when $p < 0.05$.

RESULTS

The 75 MRSA evaluated were susceptible to vancomycin with MICs ≤ 2 µg/mL. (BM): 2 µg/mL. (4%), 1 µg/mL. (50.66%), 0.5 µg/mL. (42.66%), and 0.25 µg/mL. (2.66%). The MIC_{50} and MIC_{90} were both 1 µg/mL. All isolates were susceptible to vancomycin for agar dilution screening. The MicroScan panel used had four dilution points (16, 8, 4 and 2 µg/mL.) and all isolates presented MICs ≤ 2 µg/mL.

The E-test® was statistically similar to BM ($p = 0.777$). However, PROBAC® and M.I.C.E.® were both statistically different compared to the gold standard ($p < 0.001$).

For the E-test® analysis, two approaches were used: the first one used the gross values; for the second approach, CLSI breakpoints for BM were used to evaluate the E-test® (i.e. an E-test MIC of 3 was, for this approach, considered 4 µg/mL.). This data are shown in Table 1.

The agreement among evaluated methods and BM was also evaluated, considering each MIC value to observe if the performance of the methods depended on the MIC value, as shown in Table 2.

Considerable heterogeneous performance was observed in different MIC values. In MRSA isolates with a vancomycin MIC of 0.25 µg/mL., the E-test® and M.I.C.E.® presented values at least 1-fold higher than BM for all isolates; which was also observed in most isolates with a MIC of 0.5 µg/mL.. However, for a MIC of 1 and 2 µg/mL., higher agreements for both strip-based methodologies (Table 2) were observed. For these methods, discordant results showed MICs 1-fold higher than BM for MIC 1 µg/mL.. On the other hand, for MIC 2 µg/mL., all discordant results presented MICs lower than the gold standard method. Regarding the commercial panel PROBAC®, better performances were observed in lower MICs (0.25 and 0.5 µg/mL.). For MICs 1 and 2 µg/mL., discordance was a major concern (Table 2).

In terms of global agreement with the gold standard, the E-test® had higher concordance (40%) and it was the only one statistically similar to BM, followed by PROBAC® (36%), which had a higher number of major errors (58.66%). Minor errors were mostly observed for M.I.C.E.® strips (67.99%).

DISCUSSION

The therapeutic failure related to vancomycin is well established, especially regarding to MIC creeps[13]. Most hospitals report estimated vancomycin MICs through automated methods. However, different authors show evidence that MIC creeps are not accurately detected by automated systems[4,5]. The failure during vancomycin therapy is particularly associated to pharmacokinetic and pharmacodinamic characteristics of the drug, which needs a ratio area under the curve/MIC higher than 400 to obtain therapeutic success. When isolates present a MIC of 2 µg/mL., this ratio is hard to achieve, once serum vancomycin concentration should be 15 and 20 µg/mL.[16].

In this study, the E-test® was, in general, the method with a higher agreement with BM, presenting the most homogeneous performance in different MIC values. The commercial panel PROBAC® presented better performance in lower MIC data regarding these panels, which is extremely relevant, considering the absence of previous information on

Table 1
Distribution of MRSA according to MIC values and methodologies

MIC (µg/mL)	Methodology % (*n*)				
	Broth microdilution	PROBAC®	E-test®	E-test®***	M.I.C.E.®
0.25	2.66% (2)	18.66% (14)	1.33% (1)	1.33% (1)	1.33% (1)
0.38*	NA**	NA**	5.33% (4)	NA**	NA**
0.50	42.66% (32)	68% (51)	14.66% (11)	20% (15)	2.66% (2)
0.75*	NA**	NA**	25.33% (19)	NA**	NA**
1.00	50.66% (38)	13.33% (10)	30.66% (23)	56% (42)	38.66% (29)
1.50*	NA**	NA**	22.66% (17)	NA**	NA**
2.00	4% (3)	0% (0)	0% (0)	22.66% (17)	57.33% (43)
	100% (75)	100% (75)	100% (75)	100% (75)	100% (75)

MRSA: Methicillin-resistant *Staphylococcus aureus*; MIC: Minimal Inhibitory Concentration; *MICs value observed only on E-test® strip; **NA = not applicable; ***CLSI breakpoints for BM were used for the evaluation of E-tests® results.

Table 2
Agreement (%) among methods and BM, according to vancomycin MIC values

Broth microdilution MIC (µg/mL)	Methodology	Agreement % (n)	Lower MIC* % (n)	1X higher** MIC % (n)	2X higher*** MIC % (n)
0.25	PROBAC®	50.00% (1)	0.00% (0)	50.00% (1)	0.00% (0)
	E-test® *	0.00% (0)	0.00% (0)	50.00% (1)	50.00% (1)
	M.I.C.E.®	0.00% (0)	0.00% (0)	0.00% (0)	100.00% (2)
0.50	PROBAC®	59.37% (19)	31.25% (10)	9.38% (3)	0.00% (0)
	E-test® *	25.00% (8)	0.00% (0)	56.25% (18)	18.75% (6)
	M.I.C.E.®	3.12% (1)	3.12% (1)	28.13% (9)	65.63% (21)
1.00	PROBAC®	18.42% (7)	81.58% (31)	0.00% (0)	0.00% (0)
	E-test® *	55.26% (21)	18.42% (7)	26.32% (10)	0.00% (0)
	M.I.C.E.®	47.37% (18)	2.63% (1)	50.00% (19)	0.00% (0)
2.00	PROBAC®	0.00% (0)	100.00% (3)	0.00% (0)	0.00% (0)
	E-test® *	33.33% (1)	66.67% (2)	0.00% (0)	0.00% (0)
	M.I.C.E.®	66.67% (2)	33.33% (1)	0.00% (0)	0.00% (0)
Global agreement	PROBAC®	36.00% (27)	58.66% (44)	5.33% (4)	0.00% (0)
	E-test® *	40.00% (30)	12.00% (9)	38.66% (29)	9.33% (7)
	M.I.C.E.®	28.00% (21)	4.00% (3)	37.33% (28)	30.66% (23)

*CLSI breakpoints for BM were used for the evaluation of E-test® results; *MICs were defined as lower than the BM; **MICs were defined as one-fold dilution higher than the BM; ***MICs were defined as two-fold dilution higher than the BM.

the performance of this method. To the authors' knowledge, this is the first study to evaluate the accuracy of MICs determined by PROBAC® panels.

On the other hand, M.I.C.E.® had better performance with higher MICs. Global agreement of M.I.C.E.® (28%) was considerably lower than that observed by CAMPANA *et al.* (2011) (76.3%). Besides, MUSHTAQ *et al.* (2010) observed elevated rates of agreement between the strips (M.I.C.E.® and E-test®), concluding that both are appropriate for clinical laboratory use. In this study, the low global agreement of M.I.C.E.® strips does not point them as accurate methods.

VAN HAL *et al.* (2012), in his meta-analysis, showed no statistical difference between mortality associated with infections caused by *S. aureus* strains and vancomycin MIC of 1.5 µg/mL and 1 µg/mL. However, mortality associated with strains presenting MIC 2 µg/mL and 1.5 µg/mL was statistically different. Therefore, the interpretation of M.I.C.E.® results is compromised, once it does not present the 1.5 µg/mL value of MIC. So, the M.I.C.E.® MIC of 2 µg/mL may, in fact, represent 1.5 µg/mL or 2 µg/mL., which could lead to therapeutic failure.

According to SWENSON *et al.* (2009) and RYBAC *et al.* (2013), the E-test® and MicroScan lead to a higher BM of MIC 1-fold. CDC recommends that the clinical laboratory should define an algorithm to determine which additional tests would be necessary to confirm an *S. aureus* as having reduced susceptibility to vancomycin. This algorithm should consider characteristics of patients and resources available in the clinical laboratory[3]. As MIC average of population may affect performance of tests, it should be considered when choosing alternative methodologies for broth microdilution.

For the MRSA isolates tested, the E-test® presented the best performance. Even though, overestimated MIC, also described by other authors, compromises the accuracy of the method. Nevertheless, these non-accurate MICs represent minor errors, which have lower impact on the treatment of patients, compared to major errors. So, this data support the use of the E-test® as a rapid and easy test.

This study has some limitations. First, the reduced number of isolates could have compromised the statistical analysis. Second, the MRSA population tested presented low MICs; studies with a different population of MRSA must be conducted to evaluate the performance of methods in strains with higher chances of leading to therapeutic failures and determining if differences in performance would also be observed.

Another point of concern is that MIC values may suffer alterations after cryopreservation. EDWARDS *et al.* (2012) demonstrated that MICs from automated systems and the E-test® were significantly lower after cryopreservation, if compared with those from the E-test® analysis, at the time of isolation, either for vancomycin and daptomycin. SCHAUMBURG *et al.* (2014) also pointed out that the prevalence of vancomycin MIC creeps may be underestimated because of the cryopreservation effect. Therefore, vancomycin MIC creeps might be lost after cryoconservation[5,16]. This variable was not considered in the study population. Further studies must be designed to reinforce previous observations.

Monitoring the occurrence of *S. aureus* with reduced susceptibility to vancomycin is a subject. For the population of MRSA tested, the E-test® presented the best performance, although with heterogeneous accuracy,

depending on MIC values. Thus, the choice of method to determine MIC values must take into consideration costs, conditions of the clinical laboratory and the characteristics of the *S. aureus* populations evaluated.

RESUMO

Avaliação de métodos na detecção da MIC de vancomicina e mudanças na acurácia relacionada a diferentes valores de MIC

INTRODUÇÃO: *Staphylococcus aureus* resistente à meticilina (MRSA) apresentando suscetibilidade reduzida à vancomicina tem sido associado à falha terapêutica. Alguns métodos utilizados por laboratórios clínicos podem não ser suficientemente precisos para detectar este fenótipo, comprometendo os resultados e o desfecho do paciente. **OBJETIVOS:** Avaliar o desempenho de métodos na detecção dos valores de MIC de vancomicina entre isolados clínicos de MRSA. **MATERIAIS E MÉTODOS:** Determinamos a Concentração Inibitória Mínima de Vancomicina para 75 MRSA isolados de pacientes do Hospital Mãe de Deus, Porto Alegre, Brasil. Utilizamos a microdiluição em caldo como técnica padrão-ouro e os seguintes métodos: tiras de E-test® (BioMérieux), tiras M.I.C.E® (Oxoid), painel comercial da PROBAC® e sistema automatizado MicroScan® (Siemens). Além disso, foi realizado o teste de triagem em ágar com 3 µg/mL de vancomicina. **RESULTADOS:** Todos os isolados apresentaram MIC ≤ 2 µg/mL.. Não houve diferença estatística entre os resultados do E-test® e da microdiluição em caldo. O painel da PROBAC® apresentou MICs, em geral, menores que o padrão-ouro (58,66% de erros maiores), enquanto que as MICs pelo M.I.C.E.® foram maiores (67,99% de erros menores). **CONCLUSÕES:** Para nossa população de MRSA, E-test® apresentou o melhor desempenho, embora com uma acurácia heterogênea, dependendo dos valores da MIC.

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