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Caracterização Fenotípica e Análise de Fatores de Virulência em *Staphylococcus saprophyticus*

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"Não é preciso ter olhos abertos para ver o sol, nem é preciso ter ouvidos afiados para ouvir o trovão. Para ser vitorioso você precisa ver o que não está visível"

Sun Tzu, ***A Arte da Guerra***

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SUMÁRIO

RESUMO

Introdução: Elevado número de espécies bacterianas pertencentes ao gênero *Staphylococcus* foram descritas como integrantes da microbiota normal do hospedeiro humano. Entretanto, processos infecciosos por micro-organismos historicamente considerados de baixa relevância, principalmente pertencentes aos *Staphylococcus* coagulase-negativos (SCoN), têm sido reportados na literatura. Além disso, entre os SCoN há um subgrupo de espécies bacterianas emergentes nas quais compartilham características fenotípicas e filogenéticas estritas com isolados de *S. saprophyticus*, tornando-se de difícil discriminação no laboratório de microbiologia clínica. Essa espécie, segundo a literatura, é o principal coco Gram-positivo de infecções do trato urinário (ITU) não-complicadas em mulheres jovens, possuindo diversas proteínas de superfície de papel reconhecido na aderência e colonização do uro-epitélio. **Objetivos:** O presente estudo teve por objetivos avaliar o desempenho dos sistemas automatizado Vitek 2 e Vitek MS na identificação de cocos Gram-positivos, com especial atenção na discriminação de espécies de *Staphylococcus* do grupo *saprophyticus*, além de caracterizar os potenciais fatores de virulência desse uropatógeno. **Materiais e Métodos**: Cento e quatro isolados bacterianos de cocos Gram-positivos foram testados quanto a acurácia do sistema Vitek 2 (automação fenotípica) e 450 isolados clínicos para o Vitek MS (MALDI-TOF MS). Amostras de *S. saprophyticus* foram avaliadas quanto à capacidade de formação de biofilme, aderência às células de linhagem uro-epitelial T-24 e frequência de genes associados à adesão (*aas, uafA, sdrI* e *ssp*), além de caracterização fenotípica e perfil de suscetibilidade aos antimicrobianos. **Resultados e Discussão**: O sistema Vitek 2 corretamente identificou as seguintes amostras bacterianas: 73,7% *Enterococcus*, 76,7% *Staphylococcus* e 77,8% *Streptococcus*. A identificação em nível de espécies para os micro-organismos comumente isolados, como *S. aureus* e *E. faecalis*, foi de 100%. Contudo, para as espécies menos frequentes, a acurácia foi aquém do recomendado, principalmente para SCoN. O desempenho do sistema Vitek 2 na discriminação de cepas de *Staphylococcus* do grupo *saprophyticus* foi semelhante ao observado com os SCoN (86,7%). Diferentemente do método automatizado baseado em provas fenotípicas de identificação, a metodologia MALDI-TOF obteve elevada acurácia na identificação de micro-organismos cocos Gram-positivos (97,8%). Apesar de elevado número de amostras terem apresentado perfil atípico pelo método fenotípico referência (60,4%), 99% dos *S. saprophyticus* foram identificados corretamente pelo Vitek MS, bem como outras espécies pertencentes ao grupo. A formação de biofilme foi prevalente

nos isolados de *S. saprophyticus* (98,5%), com a maioria categorizados como forte e moderadamente aderentes (78,6%), além de elevada frequência de potenciais fatores de virulência associados à aderência como *aas* (98,5%), *uafA* (100%) e *ssp* (98,5%). Além disso, apesar dessas amostras mostrarem aderência à linhagem uro-epitelial T-24, não foi evidenciada diferenças estatisticamente significativa com isolados de *E. faecalis*, outro uropatógeno comum em ITU não-complicadas. Embora as taxas de resistência tenham se mostrado baixas, especial atenção deve ser dada a reduzida suscetibilidade à trimetroprim-sulfametoxazol (10,9%) e norfloxacina (12%), antimicrobianos comumente utilizados no tratamento de ITU. **Conclusão:** A partir dos resultados obtidos, foi evidenciada elevada variabilidade fenotípica entre isolados de *Staphylococcus saprophyticus*, podendo acarretar em dificuldades na identificação desses patógenos. Como alternativa, a metodologia MALDI-TOF mostrou elevada especificidade na identificação de cocos Gram-positivos, inclusive os pertencentes ao grupo *saprophyticus*. A caracterização desses isolados evidenciou a presença de um perfil de fatores de virulência associados à adesão no trato urinário, demonstrando o potencial uropatogênico de isolados de *S. saprophyticus*.

Palavras-Chave: Cocos Gram-positivos, *Staphylococcus saprophyticus*, Identificação bacteriana, Aderência ao uro-epitélio.

ABSTRACT

Introduction: Many *Staphylococcus* bacterial species were described as members of normal flora in humans. However, infectious diseases by microorganisms that historically were reported of low virulence, mainly Coagulase-negative Staphylococcus (CoNS), have been described. Furthermore, among CoNS there are emerging bacterial species that share phenotypic and phylogenetic profile with *S. saprophyticus* strains, the mainly Gram-positive cocci isolated from non-complicated urinary tract infections (UTI) in young and sexually active women. These strains have several surface proteins with important role in adhesion and colonization of uro-epithelial tissue. **Objetives**: The aim of the study was evaluate the performance of automated system Vitek 2 and Vitek MS in bacterial identification of Gram-positive cocci and *Staphylococcus saprophyticus* group, as well as to evaluate the virulence factors in these strains. **Material and Methods**: One hundred four clinical strains were evaluated as to accuracy of Vitek 2 and 450 isolates to Vitek MS (MALDI-TOF) systems. Biofilm formation, adherence assay in uro-epithelial T-24 tissue cells and adherence associated factors (*aas, uafA, sdrI* e *ssp*) were performed in *S. saprophyticus* isolates, beyond phenotypic characterization and antimicrobial susceptibility profile. **Results and Discussion**: The Vitek 2 system correctly identified the following strains: 73,7% *Enterococcus*, 76,7% *Staphylococcus* and 77,8% *Streptococcus*. All the commonly bacterial strains, as *S. aureus* and *E. faecalis*, were correctly identified in species level. However, low accuracy was found in CoNS, similarly to *Staphylococcus saprophyticus* group (86.7%). The MALDI-TOF agreement rate in the Gram-positive cocci identification was 97.8%. Although of high number of strains have shown atypic profile by reference methodology (60.4%), 99% of *S. saprophyticus* isolates were correctly identified by Vitek MS, as well as others bacterial species of the *S. saprophyticus* group. The biofilm formation was found in 98.5% of *S. saprophyticus* strains, mainly categorized as strong and moderate phenotypes (78.6%), and elevated frequency of potential adherence factors was found – *aas* (98,5%), *uafA* (100%) and *ssp* (98,5%). Moreover, although these bacterial strains have shown adhesion to uro-epithelial T-24 cells, there was no statistical difference among *E. faecalis* isolates. Although low resistance rates was found in *S. saprophyticus* strains, elevated non-susceptibility (intermediate or resistance phenotypes) to trimethoprim-sulfamethoxazole and norfloxacin were found (10.9% and 12%, respectively), antimicrobial agents commonly used to treat UTI. **Conclusion**: Our results demonstrated high phenotypic variability among *Staphylococcus*

saprophyticus strains, which may to dificult the bacterial identification of theses pathogens. Instead, the MALDI-TOF metodology showed high specificity in the Gram-positive cocci, including S. saprophyticus group. The bacterial caracterization revealed a presence of virulence factors profile associated to adhesion in urinary tract by *S. saprophyticus*.

Keywords: Gram-positive Cocci, *Staphylococcus saprophyticus*, Bacterial Identification, uro-epithelial adherence.

INTRODUÇÃO

O Gênero *Staphylococcus*

O gênero *Staphylococcus* constitui um dos principais grupos bacterianos presentes na microbiota de seres humanos, sendo encontrado na pele (GAO et al., 2010), conjuntiva (DONG et al., 2011), cavidades nasais (FRANK et al., 2010), intestinos (SANNASIDDAPPA et al., 2011) e o trato urinário em menor proporção (RUPP; SOPER; ARCHER, 1992). Esses micro-organismos se apresentam como cocos Gram-positivos, distinguindo-se de outros gêneros também presentes na microbiota com morfologia celular semelhante (*Streptococcus* e *Enterococcus*) pela prova positiva da catalase. Ao menos, 60 espécies e subespécies de *Staphylococcus* foram descritos na literatura, além de apresentar 15 subgrupos genealogicamente e filogeneticamente estritamente relacionados (LAMERS et al., 2012).

O espectro de doenças causadas por representantes do gênero é principalmente devido ao caráter oportunista das infecções. Apesar de pertencer a microbiota, esses micro-organismos passam a expressar potencial patogênico devido a sua translocação do sítio habitual de colonização, acarretando em importantes processos infecciosos. A espécie *S. aureus* é a mais virulenta do gênero, podendo acometer indivíduos adultos imunocompetentes sem nenhum fator de risco, levando ao desenvolvimento de abcessos, celulite, infecções de pele e tecidos moles, osteomielite, fascite necrotizante entre outras doenças (DHANOA et al., 2012). Esse patógeno detém um poderoso arsenal de virulência, que permite que isolados bacterianos possam se ancorar, persistir, evadir e destruir as defesas do hospedeiro. Além disso, a aquisição de genes de resistência a diversas classes de antimicrobianos, especialmente aos beta-lactâmicos pela aquisição do gene *mec*A que codifica uma proteína ligadora de penicilina de baixa afinidade (do inglês *penicillin-binding protein* PBP2A) (GORDON;

LOWY, 2008), tornou-se um problema de saúde pública, devido a escassez de opções terapêuticas para o tratamento de infecções severas adquiridas no meio hospitalar e comunitário (MERTENS et al., 2012).

Isolados bacterianos de *S. epidermidis,* a principal espécie do grupo dos *Staphylococcus* coagulase-negativos (SCoN), se encontram amplamente distribuídos como parte da microbiota normal de hospedeiros humanos, podendo ser comumente encontrados colonizando axilas, cabeça e narinas. Diferentemente de *S. aureus*, que apresenta mecanismos de virulência específicos que permitem a infecção, *S. epidermidis* é descrito como um patógeno oportunista que pode causar doenças significativas quando a barreira epitelial do hospedeiro apresenta algum comprometimento, principalmente pelo uso invasivo de dispositivos médicos como próteses articulares e cateteres intravasculares. Geralmente, essas infecções são de caráter crônico e relacionadas à formação de biofilme, caracterizando o principal fator de virulência dessa espécie (OTTO, 2012).

Como um importante SCoN recuperado a partir de hemoculturas, *S. haemolyticus* é uns dos principais patógenos emergentes que apresenta perfil de multirresistência aos antimicrobianos, inclusive aos beta-lactâmicos pela aquisição do gene *mec*A (BARROS et al., 2012), com casos de resistência a classe das oxazolidinonas (linezolida – uma das opções terapêuticas para infecções por bactérias Gram-positivas, inclusive às resistentes aos beta-lactâmicos) relatados na literatura (GUPTA et al., 2012). É também descrito como agente etiológico em infecções acometendo pacientes imunossuprimidos, uma vez que é altamente prevalente no ambiente hospitalar (CAVANAGH et al., 2012). Em estudo recente conduzido por Barros e coloboradores (2012) em isolados clínicos de *S. haemolyticus* recuperados de pacientes atendidos em um hospital do Rio de Janeiro, Brasil, foi demonstrada alta variabilidade genética entre as cepas, com significante prevalência de resistência aos antimicrobianos beta-lactâmicos e a outras classes de antibióticos (87% e 75%,

respectivamente). Essa reduzida susceptibilidade aos antimicrobianos pode representar uma dificuldade no tratamento de infecções por *S. haemolyticus* multirresistentes, inclusive com heterorresistência à classe dos glicopeptídeos representados pela vancomicina e teicoplanina, acarretando em óbitos por essa espécie em endocardites adquiridas tanto na comunidade como no meio hospitalar (FALCONE et al., 2007).

Diferentemente de outras espécies de SCoN recuperadas de infecções em humanos, nas quais apresentam baixa virulência, *S. lugdunensis* é descrito como um micro-organismo oportunista que detém características semelhantes ao *S. aureus*, devido aos mecanismos de patogenicidade apresentados por seus isolados (FRANK; DEL POZO; PATEL, 2008). Embora isolados bacterianos de *S. lugdunensis* apresentem elevada susceptibilidade aos antimicrobianos (TAN; NG; HE, 2008), seus fatores de virulência como hemolisinas, proteínas de ligação à matriz extracelular (fibrinogênio), plaquetas e células endoteliais, além da capacidade de formar biofilme (FRANK; DEL POZO; PATEL, 2008), tornam essa espécie capaz de causar infecções significativas em diversos sítios anatômicos. Abcessos e infecções de feridas (PAPAPETROPOULOS; PAPAPETROPOULOU; VANTARAKIS, 2012), endocardites infecciosas (CHUNG et al., 2012; LIANG et al., 2012; TSAO et al., 2012) e mais recentemente fascite necrotizante (HUNG et al., 2012) são exemplos de processos infecciosos de etiologia por *S. lugdunensis*, exemplificando o papel dessa espécie de SCoN como um importante patógeno em humanos.

Outras espécies de SCoN que podem estar envolvidas em processos infecciosos invasivos, embora menos frequentemente isolados, são *S. warneri* e *S. hominis*. *Staphylococcus warneri* é descrito na literatura como um patógeno raro, entretanto, quando recuperado a partir de infecções relacionadas ao uso de dispositivos de acesso ao sistema nervoso central (*shunt*), pode tornar-se potencialmente perigoso para o paciente, tanto àqueles imunossuprimidos quanto imunocompetentes (TORRE et al., 1992). Além disso, é um

importante agente etiológico de bacteremias pediátricas adquiridas no leito hospitalar, estritamente relacionado ao uso de dispositivos intravasculares (BUTTERY et al., 1997). Embora seja incomum seu isolamento de endocardites infecciosas, Arslan et al (2011) relata que a recorrência de *S. warneri* isolados a partir desse tipo de acometimento pode ser atribuída à capacidade de formar biofilme em dispositivos médicos, dando acesso ao patógeno para diferentes sítios anatômicos do hospedeiro, podendo desencadear artrite séptica, por exemplo, como reportado previamente (LEGIUS et al., 2012).

S. hominis é uma bactéria comensal do corpo humano, sendo considerado no passado contaminante quando isolado de culturas microbiológicas, contudo, atualmente é descrito como um patógeno oportunista e nosocomial que pode ocasionar infecções importantes em pacientes imunossuprimidos (JIANG et al., 2012). A literatura descreve o espectro de doenças de *S. hominis* principalmente em sepse, infecções articulares (ROY et al., 2011) e bacteremias – inclusive com micro-organismos resistentes a linezolida (MAZZARIOL et al., 2012).

Embora espécies como *S. epidermidis* e *S. aureus* possam ser recuperados de uroculturas, geralmente esse achado laboratorial é secundário a uma outra condição clínica como refluxo vesico-ureteral, bacteremia, infecção sistêmica ou em pacientes obstétricos, não representando uma infecção do trato urinário (ITU) verdadeira (AL MOHAJER; DAROUICHE, 2012; SABHARWAL, 2012; UPADHYAYULA; KAMBALAPALLI; ASMAR, 2012). Dentre o gênero *Staphylococcus*, *S. saprophyticus* é a única espécie associada à infecções do trato urinário (ITU) não complicadas em hospedeiros humanos. Outrossim, é o segundo uropatógeno mais prevalente em ITU, acometendo principalmente um grupo restrito, a saber, mulheres jovens e sexualmente ativas. Esse micro-organismo apresenta diversos fatores de virulência relacionados à adesão e a persistência ao nicho urinário, além de um sistema de transporte de íons capaz de manter a viabilidade celular no micro-ambiente estressante da urina (KURODA et al., 2005; RAZ; COLODNER; KUNIN,

2005).

Uma vez que diferentes espécies bacterianas pertencentes ao gênero *Staphylococcus* podem desencadear processos infecciosos importantes em seres humanos, o diagnóstico laboratorial em nível de espécie torna-se crucial para o manejo e tratamento dos pacientes acometidos por esses patógenos. As identificações bacterianas são baseadas em ensaios bioquímicos convencionais, a partir de um padrão de utilização de determinados substratos, atividades enzimáticas e perfis de susceptibilidade. Entretanto, esses métodos mostram-se imprecisos devido a variação fenotípica e perfil semelhante entre diferentes espécies de *Staphylococcus*, principalmente os do grupo SCoN (HIROTAKI et al., 2011).

Estudos demonstram que determinadas espécies de *Staphylococcus* estão filogeneticamente relacionadas, podendo ser divididas em distintos subgrupos pela similaridade entre genes conservados como, por exemplo, o 16S rRNA (TAKAHASHI; SATOH; KIKUCHI, 1999). Além disso, podem compartilhar um perfil fenotípico semelhante, levando a uma identificação equivocada entre espécies com potenciais patogênicos distintos, acarretando no subdiagnóstico de determinados micro-organismos emergentes, como os *Staphylococcus* do grupo *saprophyticus.*

Staphylococcus **do Grupo** ***saprophyticus***

Baseado na homologia de sequências nucleotídicas do gene *16S rRNA*, Takahashi e colaboradores (1999) descreveram um grupo de espécies de *Staphylococcus* estritamente relacionadas à espécie *S. saprophyticus*: *Staphylococcus arlettae, Staphylococcus cohnii, Staphylococcus equorum, Staphylococcus gallinarum, Staphylococcus kloosii* e *Staphylococcus xylosus.* Estudo posterior demonstrou que a espécie *Staphylococcus succinus* e o grupo Saprophyticus apresentavam relações filogenéticas estreitas, determinada pela

homologia com o gene conservado *dnaJ* (SHAH et al., 2007). Atualmente, baseado em método de filogenia com múltiplas sequências nucleotídicas de genes constitutivos presentes no gênero, como *16S rRNA* , *dnaJ*, *rpoB* e *tuf*, 11 espécies pertencem ao grupo Saprophyticus, com 3 novos integrantes: *Staphylococcus nepalensis, Staphylococcus massiliensis* e *Staphylococcus pettenkoferi* (LAMERS et al., 2012).

Entretanto, fenotipicamente, outras espécies podem fazer parte dos *Staphylococcus* do grupo *saprophyticus*, devido a principal característica da espécie – a resistência a novobiocina. Catorze espécies de *Staphylococcus* foram descritas apresentando esse perfil, como *S. arlettae, S. cohnii* subs *cohnii* e *S. cohnii* subs *urealyticus, S. equorum* subs *equorum, S. fleurettii, S. gallinarum, S. hominis* subs *novobiosepticus, S. kloosii, S. lentus, S. nepalensis, S. saprophyticus* subs *bovis* e *S. saprophyticus* subs *saprophyticus, S. sciuri* subs *carnaticus, S. sciuri* subs *rodentium* e *S. sciuri* subs *sciuri, S. stepanovicii, S. succinus* subs *casei* e *S. succinus* subs *succinus* e *S. xylosus* (BANNERMAM; PEACOCK, 2007).

Dentre essas espécies de *Staphylococcus*, a maioria foi descrita como agente etiológico em infecções em humanos, com exceção de *S. fleurettii* e *S. stepanovicii*, micro-organismos nos quais foram relatados a partir de isolamento de alimentos como queijos de leite de cabra e de pequenos mamíferos silvestres, respectivamente (VERNOZY-ROZAND, 2000; HAUSCHILD; STEPANOVIĆ; ZAKRZEWSKA-CZERWIŃSKA , 2010).

Infecções por *S. arlettae*, *S. lentus*, *S. kloosii* e *S. gallinarum* geralmente acometem pacientes com condições clínicas prévias, como anormalidades anatômicas vasculares, imunossuprimidos ou com co-infecções virais, sendo raramente reportados como patógenos primários (KARACHALIOS et al., 2006; PEER et al., 2011; DINAKARAN et al., 2012; MORFIN-OTERO et al., 2012). Embora *S. equorum, S. nepalensis* e *S. succinus* tenham sido descritos e isolados primeiramente como patógenos em grande mamíferos – como os equinos e cabras – e de alimentos, respectivamente; poucas cepas foram encontradas em materiais

clínicos relevantes de humanos (NOVÁKOVÁ et al., 2006a; NOVÁKOVÁ et al., 2006b), diferentemente de isolados de *S. sciuri*, no qual tem sido associado a infecções como endocardites, ITU e infecções de feridas (COIMBRA et al., 2011).

Dentre as espécies pertencentes ao grupo Saprophyticus, *S. cohnii, S. saprophyticus* e *S. xylosus* são descritos como os principais micro-organismos recuperados de infecções em humanos. A espécie de *S. cohnii* apresenta características singulares que permitem a colonização em humanos, principalmente na pele como parte da microbiota normal do hospedeiro (WALDON; SOBIŚ-GLINKOWSKA; SZEWCZYK, 2002; D'AZEVEDO et al., 2008). Apesar de infecções oportunistas por *S. cohnii* serem descritas em pacientes imunossuprimidos, esses micro-organismos apresentam baixo potencial patogênico comparado com outros SCoN (BASAGLIA et al., 2003). Além disso, resistência aos antimicrobianos beta-lactâmicos pela aquisição do gene *mec*A e a linezolida já foram encontrados em isolados clínicos de *S. cohnii* (PETINAKI et al., 2009; ZONG; LÜ, 2010; GARZA-GONZÁLEZ, 2011).

Staphylococcus xylosus é uma bactéria comensal geralmente encontrada na pele e membranas mucosas de mamíferos. Além disso, é naturalmente encontrada no ambiente e em produtos alimentícios como carnes cruas e leite, sendo muito utilizada em processos de fermentação. Como um patógeno, já foi descrito em endocardites, pielonefrites e ITU, além de ser recuperado de abscesso cerebral otogênico (AKHADDAR et al., 2010) e, mais recentemente, eritema associado à septicemia (GIORDANO et al., 2012).

Devido as características singulares de isolados de *S. saprophyticus*, especial atenção será dada à descrição dessa espécie como um importante membro do grupo com potencial patogênico em hospedeiros humanos, como segue.

Staphylococcus saprophyticus

Dentre os cocos Gram-positivos, as infecções por *S. saprophyticus* é a segunda causa de infecções de trato urinário adquiridas na comunidade, afetando principalmente um grupo restrito de pacientes: mulheres jovens e sexualmente ativas (MINARDI et al., 2011). Esse micro-organismo apresenta características singulares que permitem que seja descrito como um dos únicos representante do gênero *Staphylococcus* capaz de levar a quadros de cistite por seus mecanismos de virulência. Outros isolados bacterianos, principalmente do grupo dos SCoN, podem causar ITU, entretanto, o processo infeccioso é freqüentemente correlacionado com pacientes hospitalizados que estão em uso de dispositivos médicos, como o uso de cateteres urinários (KURODA et al., 2005).

A espécie em questão é frequentemente isolada da região perianal, o que faz com estes micro-organismos estejam mais envolvidos em infecções do trato urinário, principalmente em mulheres jovens e sexualmente ativas (WIDERSTRÖM et al., 2012). Além disso, é o segundo mais importante, seguido somente por *Escherichia coli*, causador de ITU não complicadas em mulheres. Algumas complicações mais severas podem ocorrer, como pielonefrite aguda, septicemia, nefrolitíase e endocardite. No sexo masculino, ITU por *S. saprophyticus* são menos frequentes, entretanto, podem acometer homens de todas as idades. Nestes pacientes, podem ocorrer uretrite, epididimite e prostatite (RAZ; COLODNER; KUNIN, 2005).

Patogênese das Infecções por *Staphylococcus saprophyticus*

Historicamente, os SCoN eram considerados contaminantes quando isolados a partir de material clínico, analogamente aos isolados de *S. saprophyticus*. Pouco se sabia dos

mecanismos patogênicos desse micro-organismo, sabendo-se apenas que era recuperado de ITU não complicados como patógenos primários e que atingiam um grupo restrito de pacientes, a saber, mulheres jovens (GILLESPIE et al., 1978). Com a evolução dos estudos científicos desse uropatógeno, diversos fatores foram correlacionados para colonização por esse micro-organismo, como intercurso sexual recente, variação sazonal e colonização do trato gastro-intestinal (RUPP; SOPER; ARCHER, 1992). Essas infecções também se assemelhavam às infecções por bactérias Gram-negativas, na qual os sujeitos de pesquisa experimentavam colonização recorrente por diferentes cepas de *S. saprophyticus* (RUPP; HAN; GOERING, 1995). Esses estudos, entretanto, pouco elucidaram os mecanismos e determinantes de virulência envolvidos no processo infeccioso por esse patógeno.

O potencial patogênico de isolados de *S. saprophyticus* é decorrente da presença de diversas proteínas de superfície que medeiam a aderência da célula bacteriana à célula hospedeira. Essa interação permite que o micro-organismo se ancore principalmente nas células uro-epiteliais, induzindo um processo inflamatório no tecido, caracterizando o quadro de cistite.

Diversas determinantes de virulência foram descritos para a espécie. Na análise comparativa do genoma de *S. saprophyticus* com outros dois principais representantes do gênero *Staphylococcus* (*S. aureus* e *S. epidermidis*), foi verificado que este uropatógeno possui em seu cromossomo informações que revelam a presença de proteínas ancoradas à parede celular e um sistema de transporte de íons que facilita a adaptação ao ambiente urinário. Além disso, a atividade da enzima urease de isolados de *S. saprophyticus* é significativamente maior comparada às outras espécies de *Staphylococcus*, sendo considerada um importante fator de virulência para a persistência de infecções no trato urinário. Portanto, o *S. saprophyticus* se adaptou especificamente para o ambiente urinário, expressando fatores de aderência e um notável crescimento nesse meio (KURODA et al., 2005).

Fatores de Virulência Associados à Aderência

Adesinas Associadas à Adesão ao Uro-epitélio e às Proteínas da Matriz Extracelular

Sendo de fundamental importância ao processo infeccioso à aderência dos isolados bacterianos aos tecidos do hospedeiro, especificamente ao uro-epitélio, diversas proteínas bacterianas de superfície foram descritas como determinantes de aderência e virulência de *S. saprophyticus*, como UafA, Aas, Ssp e SdrI (KLEINE; GATERMANN; SAKINC, 2010).

O processo de aderência da célula bacteriana ao tecido do hospedeiro é mediado por proteínas de superfície celular chamadas de *Microbial Surface Components Recognizing Adhesive Matrix Molecules* (MSCRAMMs). Dessas moléculas adesivas, são descritas aquelas que estão covalentemente ancoradas ao peptidioglicano da parede celular de patógenos Gram-positivos por um motivo de aminoácidos LPXTG. Interessantemente, no mesmo estudo que realizou a análise genômica comparativa do *S. saprophyticus* com *S. aureus* e *S. epidermidis*, somente uma proteína foi encontrada tendo essas características, a *uro-adherence factor* A (UafA), sugerindo que esse isolado apresenta características distintas de colonização ao tecido do hospedeiro (MATSUOKA et al., 2011; KURODA et al., 2005).

A proteína UafA é uma hemaglutinina que contribui significativamente à aderência de isolados de *S. saprophyticus* às células uro-epiteliais do trato urinário, uma vez que nesse micro-ambiente as células bacterianas estão submetidas a intenso estresse devido ao fluxo de urina. Entretanto, os mecanismos moleculares de interação entre receptor e ligante do UafA não foram bem elucidados, acreditando-se que fosse uma molécula que agia como receptor de fibronectina no hospedeiro humano (GATERMANN; MEYER, 1994). Posteriormente, por

meio de ensaio de aderência e análise cristalográfica, a UafA foi descrita como uma molécula consistindo de 3 domínios, na qual não medeia a aderência por moléculas de colágeno, fibronectina ou de proteínas, estando sua molécula ligante provavelmente composta de baixo peso molecular como sacarídeos e lipídios (MEYER; WENGLER-BECKER; GATERMANN, 1996; KLEINE; GATERMANN; SAKINC, 2010; MATSUOKA et al., 2011).

A elucidação do papel de outras proteínas de superfície na patogênese dos isolados de *S. saprophyticus* tornou-se importante após a evidência de que isolados clínicos que não expressam hemaglutinação ainda mantém seu potencial virulento de causar infecções e ligar-se a moléculas de colágeno. Outra proteína MSCRAMM com motivo LPXTG foi descrita para *S. saprophyticus*, com repetições de serina/aspartato (*serine-aspartate repeat*-SdrI), na qual desempenha essas características citadas. Essa molécula de superfície apresenta estrutura similar a outras proteínas da família Sdr, com características típicas de proteínas de superfície encontradas em micro-organismos Gram-positivos. Em mutantes *knockout* para SdrI, foi evidenciado decréscimo significativo na ligação a moléculas de colágeno, comparado com a cepa selvagem. Esse resultado indica que o SdrI seria o responsável pela ligação a moléculas de colágeno em *S. saprophyticus*, uma proteína da matriz extracelular. Contudo, devido a baixa prevalência do gene *sdr*I em isolados clínicos, não ficou evidente se verdadeiramente a presença dessa proteína de superfície aumentaria a virulência ou apenas seria mais um caso de uma adesina redundante presente em isolados de *Staphylococcus* spp. (SAKINC; KLEINE; GATERMANN, 2006).

Interessantemente, isolados SdrI positivos apresentam capacidade de ligação a moléculas de fibronectina, mesmo que a estrutura dessa proteína de superfície não contenha uma região típica das proteínas ligantes dessa proteína da matriz extracelular (SAKINC et al., 2009). Em estudo de modelo animal, foi verificado que, embora a presença de SdrI não seja necessária para a colonização inicial no trato urinário, essa proteína é requerida para a

persistência dos isolados de *S. saprophyticus* em órgãos como a bexiga e os rins (KLINE et al., 2010).

Em bactérias de importância clínica como as Gram-negativas, *Streptococcus* e *S. aureus*, é reconhecido o papel de proteínas de superfície na aderência em células eucarióticas. Já em micro-organismos pertencentes ao grupo do SCoN, estudos tem sido conduzidos principalmente a fim de verificar a contribuição da formação de biofilme e produção de cápsula na virulência dessas cepas, fatores que estruturalmente são constituídos de polímeros de carboidratos. Contudo, em isolados de *S. saprophyticus*, foi descrito estruturas filamentares protéicas associadas à superfície celular, em grande quantidade, de 95 kDa denominada de proteína associada a superfície de *S. saprophyticus* (do inglês *S. saprophyticus surface-associated protein* - Ssp) que poderia ser um candidato adicional a fator de aderência a células eucarióticas de hospedeiros humanos, mesmo não apresentando atividade de hemaglutinação (GATERMANN et al., 1992).

Embora tenha sido sugerido que Ssp apresentasse função de aderência, estudos posteriores demonstraram que esse polipeptídeo não apresenta propriedades adesivas às proteínas da matriz extracelular como fibronectina, fibrinogênio, colágeno e a laminina. Atualmente, em estudo que realizou clonagem, sequenciamento e ensaio de atividade de Ssp, Sakinc e colaboradores (2005) concluíram que a maior proteína de superfície de *S. saprophyticus* é uma enzima da família das lipases, uma vez que a sua seqüência gênica apresentou homologia com lipases estafilocócicas. Além disso, é produzida em grandes quantidades por essa espécie de *Staphylococcus* e não apresenta uma função específica de adesina. Pode, como sugerido pelos autores, contribuir para a persistência do micro-organismo por promover uma fonte de energia ou facilitar a aderência, já que as evidências mostram que é expressa *in vivo* e pode apresentar função promovendo o crescimento bacteriano ou como um fator de patogênese em isolados de *S. saprophyticus* (SAKINC et al.,

2005).

Semelhantemente ao Ssp, foi descrito em isolados de *S. saprophyticus* outra proteína expressa em grande quantidade na superfície das células bacterianas, a Aas (*adhesin-autolysin of S. saprophyticus*). Em estudo conduzido por Gatermann, Meyer e Wanner (1992), foi estudada uma cepa bacteriana de *S. saprophyticus* Ssp-negativa, mas com atividade de hemaglutinação em eritrócitos de carneiro. Uma proteína de 160 kDa foi identificada e caracterizada funcionalmente pelos pesquisadores, apresentando atividade semelhante às hemaglutininas, contudo, não sendo inibida por anticorpos dirigidos contra a proteína Ssp (GATERMANN; MEYER; WANNER, 1992). Caracterização posterior dos tipos moleculares em que essa hemaglutinina poderia utilizar como receptor foram estudados em eritrócitos de carneiro. Interessantemente, ao contrário de muitas outras hemaglutininas, o receptor é do tipo protéico, uma vez que hemaglutinação não foi evidenciada em membranas de eritrócitos tratadas com proteinase (MEYER; MÜTHING; GATERMANN, 1997).

O conhecimento atual sobre a hemaglutinina de *S. saprophyticus* revela uma molécula multifuncional. Além de mediar a ligação a fibronectina, essa proteína apresenta forte homologia com autolisinas presentes em outras espécies bacterianas importantes do gênero, como *S. aureus* (*atl*) e *S. epidermidis* (*atlE*). Contudo, a caracterização dessa proteína de superfície mostrou diferenças importantes se comparadas com outras presentes em micro-organismos Gram-positivos. Essa molécula apresenta duas atividades distintas, de dispersão durante a divisão celular e de aderência a moléculas presentes em organismos hospedeiros. A combinação dessas atividades (de autolisina e de aderência) poderia contribuir para a virulência dos micro-organismos por liberar componentes da parede celular imunologicamente ativos e permitir a colonização e o desenvolvimento do processo infeccioso a partir da adesão a células uro-epiteliais do hospedeiro. Portanto, a proteína Aas – adesina/autolisina – de *S. saprophyticus* é um potencial fator de virulência dessa espécie,

representando uma nova classe de adesinas estafilocócicas (HELL; MEYER; GATERMANN, 1998).

Formação de Biofilme

No laboratório de microbiologia clínica, os SCoN estão entre os micro-organismos mais frequentemente isolados, especialmente por infecções adquiridas no leito hospitalar. Como integrantes da microbiota normal da pele e membranas mucosas, a diferenciação entre isolados contaminantes e os clinicamente importantes tornou-se um desafio na medicina. Epidemiologicamente, esse grupo de isolados bacterianos é um importante agente etiológico de infecções nosocomiais, especialmente em pacientes com um importante fator de predisposição a esse tipo de infecção: o uso de dispositivos médicos poliméricos como próteses e cateteres vasculares, nos quais os integrantes dos SCoN, bem como *S. aureus*, apresentam importante habilidade de colonizar (VON EIFF; PETERS; HEILMANN, 2002). Geralmente essas infecções não respondem à terapia antimicrobiana, com a necessidade de remoção do dispositivo implantado. Nesse contexto, a formação de biofilme tornou-se o principal fator que contribui ativamente para a patogenicidade de SCoN (MACK et al., 2004).

Biofilme é caracterizado como uma comunidade bacteriana organizada na qual apresenta agregação por uma matriz constituída de substância polimérica extracelular secretada pelas próprias células. Caracteriza uma adaptação procariótica que permite que bactérias possam sobreviver em ambientes hostis e colonizar novos nichos. Além disso, permite uma coordenação entre fatores fisiológicos e bioquímicos entre os micro-organismos por sinalização celular, como a expressão diferenciada de moléculas de superfície, utilização de nutrientes e fatores de virulência. Essa comunicação permite que as células bacterianas respondam fenotipicamente às mudanças ambientais estressantes as quais estão submetidas,

facilitando a sobrevivência a nível populacional (HALL-STOODLEY; STOODLEY, 2009).

Staphylococcus epidermidis é a espécie modelo no estudo de formação de biofilme no gênero. Embora não apresente um mecanismo de virulência agressivo, comparado ao *S. aureus*, este micro-organismo é um patógeno oportunista em humanos, uma vez que as infecções mostram-se de difícil tratamento e sobrecarregam o sistema público de saúde pelos custos decorrentes do manejo desses pacientes. Esse perfil de patogenicidade está intimamente relacionado à capacidade de formação de biofilme por essa espécie de *Staphylococcus* (OTTO, 2009).

O processo de formação de biofilme é um evento complexo. A adesão primária a superfícies abióticas é principalmente decorrente de propriedades hidrofóbicas encontradas na superfície da célula bacteriana. *In vivo*, as proteínas da matriz extracelular do hospedeiro medeiam essa aderência como receptores para diversas proteínas bacterianas ancoradas à parede celular, as MSCRAMM. Exemplo dessas proteínas de adesão encontradas em *S. epidermidis* são a AtlE, Bap e SdrG, que podem apresentar interação, em maior e menor grau, com fibronogênio, colágeno, fibronectina e vitronectina (OTTO, 2009).

A consolidação da aderência das células bacterianas, tanto em superfícies bióticas quanto em abióticas, depende da agregação intercelular. Em *S. epidermidis*, a secreção de uma matriz polissacarídica denominada de PIA (do inglês *polysaccharide intercellular adhesin*) – um homopolímero de poli-N-acetilglicosamina (PNAG) – é essencial ao biofilme, com importante impacto em infecções associadas a esse fator de virulência em muitos modelos animais. A biossíntese desse exopolissacarídeo é decorrente do produto gênico do operon *icaADBC* (*intercellular adhesion*), estando sujeito a influências regulatórias significativas, tanto por reguladores transcricionais de virulência presentes no próprio genoma do micro-organismo quanto por sinalização ambiental. Entretanto, cepas mutantes para *ica* podem ainda

expressar o fenótipo de biofilme, indicando que proteínas de adesão presentes na superfície da célula bacteriana e exopolissacarídeos secretados pelas mesmas participam eficientemente na formação de biofilme e contribuem com a fisiopatologia de *S. epidermidis* (OTTO, 2009; ROHDE et al., 2010).

Essa consolidação do biofilme acarreta na formação de microcolônias com estrutura tri-dimensional complexa. Contudo, a dispersão das células bacterianas enclausuradas na matriz polimérica do biofilme ocorre como uma etapa importante na patogênese desses micro-organismos. A ativação de mecanismos como a expressão de proteases e outras moléculas como DNAse, PSM (*Phenol Soluble Modulins* – um potente surfactante) e mudanças ambientais como a depleção de glicose podem induzir a dispersão, promovendo a capacidade de disseminação das células bacterianas a sítios secundários de infecção e transmissão entre hospedeiros infectados (BOLES; HORSWILL, 2011; OTTO, 2012; SADYKOV; BAYLES, 2012).

Infecções urinárias relacionadas ao uso de cateteres são uma das infecções mais comuns associadas a dispositivos médicos. A formação de biofilme nesses cateteres urinários também é uma importante causa de ITU em pacientes críticos, como aqueles em unidade de terapia intensiva, além de estar associada à elevada taxa de morbidade, mortalidade e custos ao sistema de saúde (DOHNT et al., 2011; DJERIBI et al., 2012; NICOLLE, 2012; SIDDIQ; DAROUICHE, 2012). ITUs associadas à formação de biofilme não só acometem pacientes com uso de dispositivos como cateteres urinários. Também estão correlacionadas com patologias urológicas como prostatite bacteriana crônica, cistite recorrente, pielonefrite e vaginose bacteriana. A patogênese dessas infecções é decorrente principalmente da aderência ao epitélio do hospedeiro, principalmente às células uro-epiteliais da bexiga e órgãos anexos, ocasionando infecções de caráter crônico (TENKE et al., 2012).

O espectro de micro-organismos agentes etiológicos de ITU associadas à formação de biofilme é constituído principalmente por bastonetes Gram-negativos da família *Enterobacteriaceae*, como *Enterobacter* sp, *Escherichia coli*, *Klebsiella pneumoniae, Proteus mirabilis, Serratia* sp e *Yersinia* sp (EJRNÆS et al., 2011; JACOBSEN; SHIRTLIFF, 2011; BONKAT et al., 2012; CHOE et al., 2012; HOLA; PEROUTKOVA; RUZICKA, 2012; STAHLHUT et al., 2012), além de bactérias como *Acinetobacter baumannii* e *Pseudomonas aeruginosa* (POUR et al., 2011; BONKAT et al., 2012; CHOE et al., 2012; DJERIBI et al., 2012). Contudo, dentre os micro-organismos Gram-positivos, representantes dos gêneros *Enterococcus* sp, *Staphylococcus* sp e *Streptococcus* também foram relatados na literatura (CHO et al., 2002; GAD et al., 2009; BONKAT et al., 2012; CHOE et al., 2012).

Cepas de *Enterococcus faecalis* estão entre os principais uropatógenos que causam ITU relacionadas ao uso de cateteres. Diversos fatores de virulência associados à aderência foram descritos, como a proteína Esp (*enterococcal surface protein*), GelE (*gelatinase*) e Ebp (*endocarditis- and biofilm-associated pilus*), os quais tornam os isolados bacterianos aptos para formar biofilme no trato urinário, tanto na superfície do dispositivo quanto no tecido epitelial do hospedeiro (SENO et al., 2005; SINGH; NALLAPAREDDY; MURRAY, 2007; GUITON et al., 2010; SILLANPÄÄ et al., 2010; NIELSEN et al., 2012).

Interessantemente, em isolados do gênero *Staphylococcus*, ITUs relacionadas ao uso de cateter urinário são descritas na literatura principalmente por *S. aureus* e *S. epidermidis*. Geralmente esses isolados apresentam elevada proporção do elemento genético *ica* e formação de biofilme, o que poderia indicar o importante papel desses genes como marcadores de virulência em infecções estafilocócicas associadas à cateterização urinária (CHO et al., 2002; GAD et al., 2009; GONZÁLEZ-DOMÍNGUEZ et al., 2012).

Embora *S. saprophyticus* seja a espécie dentre os cocos Gram-positivos mais

frequentemente isolada em ITUs, poucos estudos são descritos relacionando essas infecções com o uso de cateteres urinários. Em estudo de caso-controle com isolados bacterianos de *S. saprophyticus* e SCoN recuperados de ITUs, aspectos clínicos e epidemiológicos foram estudados por Hedman e Ringertz (1991). Mesmo que a sintomatologia como inflamação do trato urinário inferior, hematúria e piúria tenha sido mais comumente encontrada nos pacientes com infecção por *S. saprophyticus*, esse micro-organismo não foi associado em infecções pelo uso de cateterização urinária, diferentemente dos SCoN. Kunin e Steele (1985) previamente demonstraram essa correlação clínica descrita anteriormente em culturas de superfície de cateteres urinários de 398 pacientes com urina estéril com o intuito de determinar a flora bacteriana uretral presente nessa amostra. Nesse estudo, o micro-organismo mais comumente isolado foi *S. epidermidis*, com nenhuma cepa de *S. saprophyticus* recuperada, apesar da amostra de pacientes ter sido composta em grande proporção pelo grupo epidemiologicamente suscetível às infecções por esse uropatógeno: mulheres jovens e sexualmente ativas. A frequência baixa de isolados de *S. saprophyticus* recuperados de infecções relacionadas ao uso de cateteres urinários deixa dúbio o papel da expressão do fator de virulência biofilme, comum aos SCoN, na patogênese desse micro-organismo.

Poucos estudos de avaliação de formação de biofilme em isolados de *S. saprophyticus* são encontrados na literatura. Carbonero e colaboradores (1989) demonstraram em estudo *in vitro* com células uro-epiteliais que *S. saprophyticus* significativamente foi mais aderente que *S. epidermidis*, com esse fenômeno não sendo relacionado com a habilidade de formar biofilme por essas cepas. Além disso, a expressão de biofilme em *S. saprophyticus* parece ser um evento infrequente nesses isolados (HJELM; LUNDELL-ETHERDEN, 1991; RILEY; SCHNEIDER, 1992), embora o método *in vitro* utilizado – triagem em Ágar Vermelho Congo – tenha se mostrado menos acurado e produzível em comparação ao método de micro-titulação em placas em estudos posteriores, uma vez que detecta somente exopolissacarídeos

secretados pelas células bacterianas (MATHUR et al., 2006; TAMAI et al., 2011). *S. saprophyticus* não apresenta o operon *ica* no seu genoma e, portanto, não produz PIA (KURODA et al., 2005; PARK et al., 2010), podendo esses resultados estar determinando a sub-prevalência de biofilme em cepas de *S. saprophyticus*.

Os dados desatualizados de prevalência desse fator de virulência em isolados de *Staphylococcus saprophyticus* mostram a necessidade de se determinar a contribuição da formação de biofilme na patogênese desse micro-organismo.

Perfil de Susceptibilidade aos Antimicrobianos

Quando se pensa em estratégia de tratamento para infecções causadas por *S. saprophyticus*, deve-se entender que a resistência destes micro-organismos aos diferentes antimicrobianos é variável dependendo da população estudada. Além disso, como na maioria dos casos estes micro-organismos estão envolvidos em infecções do trato urinário não complicadas, os esquemas de tratamento são direcionados a estas infecções (MARTÍNEZ; RUIZ; PÉREZ, 2008).

O tratamento de ITU não complicada se realiza habitualmente com fosfomicina trometamol, fluoroquinolonas ou beta-lactâmicos; não é recomendado trimetoprim-sulfametoxazol como terapia empírica devido ao alto percentual de cepas resistentes (25%-30%). *S. saprophyticus* é relatado na literatura científica como normalmente sensível a todos os antimicrobianos utilizados no tratamento desse tipo de processo infeccioso. No entanto, esta afirmação possui algumas exceções. Sabe-se que fosfomicina trometamol não é eficaz *in vitro* e provavelmente também não o seja *in vivo* apesar de altas concentrações alcançadas na urina. Além disso, a utilização do ponto de corte para SCoN para oxacilina – Concentração

Inibitória Mínima (CIM) ≥ 0,5mg/l) – faz com que mais da metade das cepas isoladas de *S. saprophyticus* sejam consideradas resistentes aos beta-lactâmicos, os quais são antimicrobianos muito utilizados (principlamente amoxicilina-ácido clavulânico) para o tratamento infecções de trato urinário em pacientes ambulatoriais (MARTÍNEZ; RUIZ; PÉREZ, 2008).

Embora seja recomendado que se conheça o perfil de suscetibilidade do micro-organismo causador da infecção, geralmente, o tratamento é iniciado de maneira empírica, no qual somente características clínicas são levadas em conta. A identificação microbiológica não é realizada na rotina clínica (NABER et al., 2008).

No entanto, é importante que no caso de falha terapêutica o exame microbiológico seja realizado para que possa orientar da maneira mais adequada o tratamento de infecções urinárias.

Identificação de Cocos Gram-positivos: Grupo SCoN Resistentes à Novobiocina

Os SCoN resistentes à novobiocina compreendem um grupo heterogêneo composto de 14 espécies, dentre as quais treze apresentam perfil fenotípico bem caracterizado na literatura (Tabela 1). Atualmente, o método convencional padrão-ouro de identificação de isolados de *Staphylococcus* compreende a análise de 37 características como a expressão de determinadas atividades enzimáticas, susceptibilidade aos antimicrobianos e utilização de substratos específicos. Contudo, devido a alta variabilidade fenotípica e atipias apresentadas pelas cepas, o uso de métodos convencionais torna-se trabalhoso e pobre em acurácia (KLOOS; SCHLEIFER, 1975; BANNERMAM; PEACOCK, 2007).

Simplificações do método referência foram propostos por diferentes trabalhos

descritos na literatura (KLOOS; SCHLEIFER, 1975; PARISI; HAMORY, 1986; MONSEN et al., 1998; DE PAULIS et al., 2003; CUNHA; SINZATO; SILVEIRA, 2004; IORIO et al., 2007), com elevada acurácia na identificação de isolados de *Staphylococcus*. Contudo, a discriminação em nível de espécie ocorre principalmente para isolados bacterianos comumente recuperados no laboratório de microbiologia clínica, como *S. aureus*, *S. epidermidis*, *S. haemolyticus* e *S. saprophyticus*, além de apresentar ainda elevado número de testes e tempo de incubação prolongado para identificação, o que inviabiliza o uso desses métodos na rotina de um laboratório clínico.

A identificação presuntiva de isolados de *S. saprophyticus*, quando recuperados de cultura de urina, é baseada na resistência intrínseca ao antimicrobiano novobiocina (VICKERS; CHOPRA; O'NEILL, 2007). Entretanto, foram descritos na literatura outras espécies de *Staphylococcus* não-*saprophyticus* como agentes etiológicos de ITU ou colonizando o trato uro-genital humano, como *S. cohnii*, *S. lentus*, *S. sciuri* e *S. xylosus* (JOHN; GRAMLING; O'DELL, 1978; TSELENIS-KOTSOWILIS; KOLIOMICHALIS; PAPAVASSILIOU, 1982; MORGAN, 1983; NICOLLE; HOBAN; HARDING, 1983; BALDELLON, MÉGRAUD, 1985; ORRETT; SHURLAND, 1998; STEPANOVIC et al., 2003; STEPANOVIC et al., 2005; HIGASHIDE et al., 2008). Apesar de apresentar elevada sensibilidade diagnóstica, o teste de susceptibilidade à novobiocina não apresenta especificidade plena para a caracterização de cepas de *S. saprophyticus* (McTAGGART; ELLIOTT, 1989). Somado a alta variabilidade fenotípica e a número elevado de provas bioquímicas necessárias à identificação de outras espécies do grupo, o uso de novas metodologias, baseadas na automação de testes fenotípicos, genotípicos e proteômicos, ganhou espaço no setor de diagnóstico no laboratório de patologia clínica, permitindo a discriminação de inúmeras espécies bacterianas em tempo hábil (STAGER; DAVIS, 1992).

Atualmente, três sistemas automatizados de identificação bacteriana e determinação de

perfil de susceptibilidade estão amplamente difundidos no mercado diagnóstico: Phoenix (Becton Dickinson), MicroScan (Siemens) e Vitek 2 (bioMérieux) (WINSTANLEY; COURVALIN, 2011). Essas metodologias são baseadas na miniaturização de provas bioquímicas, permitindo a identificação em nível de espécie de diversos grupos taxonômicos em um curto intervalo de tempo, além de demandar pouco espaço físico e analistas clínicos na sua manipulação.

O sistema Vitek 2 utiliza cartões de identificação e perfil susceptibilidade específicos para micro-organismos Gram-positivos (GP) e Gram-negativos (GN). O cartão GP é composto de 43 testes bioquímicos que medem variáveis como a utilização de fontes de carbono, inibição de crescimento bacteriano, resistência e atividades enzimáticas. Entre o espectro de bactérias que podem ser identificadas pelo cartão GP, incluem-se as do gênero *Staphylococcus* (CROWLEY et al., 2012).

Uma abordagem proteômica para identificação de micro-organismos tem surgido como uma ferramenta importante no diagnóstico de doenças infecciosas. A metodologia de espectrometria de massa, especificamente MALDI-TOF (*Matrix Assisted Laser Desorption Ionization Time-Of-Flight*) permite a identificação bacteriana de modo rápido (1 a 2 minutos), preciso e reprodutível, além de apresentar uma boa relação custo-efetividade (CARBONNELLE et al., 2011; CARBONNELLE; NASSIF, 2011). Devido a simplicidade de uso, essa metodologia deixou de estar confinada em laboratórios de pesquisa e passou a ser utilizada na rotina dos laboratórios de microbiologia clínica, apresentando potencial para substituir e/ou complementar a identificação fenotípica convencional de muitos patógenos humanos, principalmente àqueles de difícil identificação e de cultivo, como bactérias anaeróbicas, fastidiosas e de crescimento lento (ex. micobactérias) (BIZZINI; GREUB, 2010; BISWAS; ROLAIN, 2013; KOK et al., 2013).

O poder discriminatório desse método é amplo, permitindo a identificação de bactérias

diretamente de fluidos biológicos como amostras de urina e hemoculturas em nível de espécie, sub-espécie, cepa e linhagem, além de identificação de toxinas bacterianas e determinados perfis de resistência aos antimicrobianos como a expressão de beta-lactamases e carbapenemases (SENG et al., 2010; STEENSELS; VERHAEGEN; LAGROU, 2011; WIESER et al., 2012). Devido ao alto poder resolutivo da técnica, foi demonstrado também que o uso do MALDI-TOF pode ser uma eficiente ferramenta para potenciais estudos epidemiológicos e de classificação taxonômica de micro-organismos (CROXATTO; PROD'HOM; GREUB, 2012).

O método para análise de amostras bacterianas é tecnicamente simples e rápido. Isolados bacterianos – cultivados em ágar ou diretamente de hemoculturas, por exemplo – são emulsionados juntamente com uma matriz (ácido alfa-ciano-4-hidroxi-cinâmico é utilizado preferencialmente para detecção de biomarcadores protéicos) que apresenta propriedade de absorção de energia no espectro de onda do ultra-violeta. Com a exposição a pulsos de energia proporcionado por uma fonte de laser, energia é transferida da matriz para moléculas não-voláteis do analito, ionizando-as numa fase gasosa. Essas moléculas são aceleradas através do tubo do espectrômetro de massa por ação de um campo eletro-magnético, sendo registrado o sinal da separação dos biomarcadores pela razão massa-carga obtido (m/z) (a aquisição do espectro de massa ocorre na faixa de moléculas de proteínas de 2000 à 20000 Da, que compreende de 60% a 70% do peso seco total da célula bacteriana). Por fim, o perfil dos biomarcadores gerado é comparado com perfis presentes em um banco de dados com cepas bem caracterizadas, reportando a provável espécie bacteriana identificada (WELKER, 2011; MURRAY, 2012).

A acurácia do sistema Vitek 2 e MALDI-TOF na identificação de isolados de *Staphylococcus* é extensivamente descrita na literatura (Tabela 2). Para as espécies bem caracterizadas como patógenos humanos, como *Staphylococcus aureus*, ambos os métodos

apresentam elevada acurácia na identificação desses micro-organismos. Entretanto, diferentemente da aproximação proteômica proporcionada pela metodologia MALDI-TOF na identificação bacteriana, o sistema Vitek 2 apresenta controversa acurácia para discriminação de cepas de SCoN.

Especificamente para isolados do grupo *saprophyticus*, estudos de Dupont et al. (2010) e Ferreira et al. (2012) relatam uma acurácia de 84,4% (n=32) e 81,2% (n=57), respectivamente, do sistema automatizado Vitek na identificação desses isolados, com erros na discriminação em nível de espécie principalmente para *S. saprophyticus* e *S. cohnii*. Em mesmo estudo, contudo, MALDI-TOF corretamente identificou 96,8% das cepas pertencentes ao grupo (DUPONT et al., 2010).

Apesar de serem conhecidas as limitações dos métodos na identificação dos SCoN – dos fenotípicos, a geração de dados irreprodutíveis e duvidosos devido a heterogeneidade da população bacteriana isolada e o metabolismo lento dessas bactérias; e dos proteômicos a ausência de novas espécies bacterianas presentes no banco de dados (IORIO et al., 2007; DUPONT et al., 2010) –, o aumento da acurácia é dependente da atualização e posterior validação das versões de softwares disponíveis dessas metodologias, permitindo um diagnóstico seguro para a prática clínica.

Tabela 1: Identificação fenotípica referência para caracterização das espécies de *Staphylococcus* coagulase-negativos resistentes à novobiocina descritas na literatura – adaptado de Kloos e Schleifer (1975) e Bannermam e Peacock (2007).

Espécie de *Staphylococcus*	Característica fenotípica*																																						
						Expressão de:																			Ácido produzido aerobicamente de:															
	Tamanho colonial	Pigmentação	Crescimento Anaeróbico	Crescimento Aeróbico	Coagulase	Fator Clumping	Nuclease Termo-estável	Hemolisinas	Catalase	Oxidase	Fosfatase Alcalina	Arginina Arilamidase	PYR	Ornitina Decarboxilase	Urease	β-Glicosidase	β-Glicuronidase	β-Galactosidase	Utilização da Arginina	Produção de Acetoína	Redução do Nitrato	Hidrólise da Esculina	Resistência à Novobiocina	Resistência à Polimixina B	D-Trealose	D-Manitol	D-Manose	D-Turanose	D-Xylose	D-Celobiose	L-Arabinose	Maltose	α-Lactose	Sacarose	N-acetil-glicosamina	Rafinose	Ribose
S. arlettae	v	+	-	+	-	-	-	-	+	-	(+)	-	-	-	-	ND	+	v	-	ND	-	-	+	ND	+	+	+	+	+	-	+	+	+	+	-	+	ND
S. cohnii subs *cohnii*	v	-	v	+	-	-	-	(v)	+	-	-	-	-	-	-	-	-	-	-	v	-	-	+	-	+	v	(v)	-	-	-	-	(v)	-	-	-	-	-
S. cohnii subs *urealyticus*	+	v	(+)	+	-	-	-	(v)	+	-	+	-	v	-	+	-	-	+	+	v	-	-	+	-	+	+	+	-	-	-	-	(+)	+	-	v	-	-
S. equorum subs *equorum*	-	-	-	(+)	-	-	-	(v)	+	-	(+)	-	-	-	+	ND	+	v	-	-	+	v	+	ND	+	+	+	v	+	(v)	+	v	v	+	v	-	ND
S. fleurettii	-	-	+	+	-	-	-	ND	+	+	v	-	-	-	-	ND	-	-	-	v	+	v	+	ND	+	ND	+	+	v	-	v	+	-	+	ND	-	ND
S. gallinarum	+	v	(+)	+	-	-	-	(v)	+	-	(+)	-	-	-	+	+	v	v	-	-	+	+	+	-	+	+	+	+	+	+	+	+	v	+	+	+	ND
S. hominis subs *novobiosepticus*	-	-	-	+	-	-	-	-	+	-	-	ND	-	-	+	-	-	-	-	v	v	-	+	ND	-	-	-	ND	-	-	-	+	v	(+)	-	-	-
S. kloosii	v	v	-	+	-	-	-	(v)	+	-	v	-	v	-	v	v	v	v	-	v	-	v	+	-	+	+	-	-	(v)	-	v	v	(v)	(±)	-	-	ND
S. lentus	-	v	(±)	(±)	-	-	-	-	+	+	(±)	-	-	-	-	+	-	-	-	-	+	+	+	-	+	+	(+)	(±)	(±)	+	v	v	v	+	v	+	ND
S. nepalensis	-	-	+	+	-	-	-	-	+	-	+	-	+	-	+	ND	+	+	-	-	+	+	+	-	+	+	+	v	+	-	+	+	+	+	+	-	ND
S. saprophyticus subs *bovis*	-	+	+	+	-	-	-	-	+	-	-	-	+	-	+	v	-	v	-	v	+	-	+	ND	+	+	-	+	-	-	-	+	-	+	+	-	-
S. saprophyticus subs *saprophyticus*	+	v	(+)	+	-	-	-	-	+	-	-	-	-	-	+	v	-	+	-	+	-	-	+	-	+	v	-	+	-	-	-	+	v	+	v	-	-
S. sciuri subs *carnaticus*	-	v	(v)	+	-	v	-	(±)	+	+	v	-	-	-	-	+	-	-	-	-	+	+	+	-	+	+	(v)	ND	+	(v)	v	(v)	(v)	+	-	-	ND
S. sciuri subs *rodentium*	v	v	(v)	+	-	+	-	(±)	+	+	v	-	-	-	-	+	-	-	-	-	+	+	+	-	(+)	+	(+)	ND	(v)	v	(v)	(v)	-	+	-	-	ND
S. sciuri subs *sciuri*	+	v	(+)	+	-	-	-	(±)	+	+	+	-	-	-	-	+	-	-	-	-	+	+	+	-	+	+	(v)	(±)	(v)	+	v	(v)	(v)	+	-	-	ND
S. succinus subs *casei*	ND	-	-	(+)	ND	ND	ND	-	+	-	±	-	-	-	+	ND	+	+	ND	-	+	+	+	ND	+	+	+	+	±	-	-	+	+	+	ND	-	ND

S. succinus subs *succinus*	ND	-	-	(+)	ND	ND	ND	-	+	-	+	-	-	-	+	ND	+	+	ND	-	-	+	+	ND	+	+	v	±	-	±	-	+	+	+	ND	-	ND
S. xylosus	+	v	v	+	-	-	-	-	+	-	v	-	v	-	+	+	+	+	-	v	v	v	+	-	+	+	+	v	+	-	v	+	v	+	+	-	v

* + : ≥90% dos isolados apresentam reação positiva para o teste; -: ≤10% dos isolados apresentam reação positiva para o teste; v: de 11% a 89% dos isolados apresentam reação positiva para o teste; resultado entre parênteses ( ): reação tardia; ±: reação fracamente positiva; ND: não determinado para a espécie; Diâmetro de ≥ 6mm é definido como reação positiva após incubação em ágar P de 34 a 35ºC por três dias e a temperatura ambiente (25ºC) por dois dias adicionais.

Tabela 2: Acurácia descrita na literatura dos sitema Vitek 2 e da metodologia MALDI-TOF na identificação de isolados de *Staphylococcus*.

Micro-organismo	Concordância na identificação bacteriana comparado aos métodos referência utilizados nos estudos								
	Vitek 2								
S. aureus	99%	99,20%	97%	ND*	100%	ND	ND	ND	98,22%
SCoN[#]	86%	90,50%	82%	88,50%	71,20%	87,50%	86%	75,20%	91,89%
Staphylococcus spp.	92,50%	95,60%	92%	ND	72,40%	ND	ND	ND	94,56%
Referência	Ligozzi et al, 2002	Spanu et al, 2003	Nonhoff et al, 2005	Layer et al, 2006	Delmas et al, 2008	Kim et al, 2008	d'Azevedo et al, 2009	Dupont et al, 2010	Chatzigeorgiou et al, 2011
	MALDI-TOF								
S. aureus	ND	ND	ND	ND	100%	ND	ND	ND	ND
SCoN	ND	93,20%	ND	ND	99,10%	ND	100%	99,30%	ND
Staphylococcus spp.	99,50%	ND	99,30%	94,30%	99,30%	88,50%	ND	ND	89,50%
Referência	Eigner et al, 2009	Dupont et al, 2010	Dubois et al, 2010	van Veen et al, 2010	Spanu et al, 2011	Benagli et al, 2011	Carpaij et al, 2011	Loonen et al, 2012	Matsuda et al, 2012

* Dado não disponível.

[#]*Staphylococcus* Coagulase-Negativos.

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JUSTIFICATIVA

O reconhecimento de espécies emergentes antes não recuperadas de infecções em humanos acarreta na necessidade de métodos diagnósticos mais acurados de identificação bacteriana, principalmente para patógenos estritamente relacionados e com alta variabilidade fenotípica, os quais são de difícil discriminação em nível de espécie.

Apesar de pertencer a um grupo de micro-organismos composto por 14 espécies, *Staphylococcus saprophyticus* é o principal representante, com o espectro de doença limitado a ITU não complicadas. Embora seja um coco Gram-positivo comumente recuperado nesse tipo de processo infeccioso, sua frequência é baixa comparado a *Escherichia coli*. Os mecanismos de patogenicidade desse uropatógeno são extensivamente descritos na literatura científica, entretanto, para *S. saprophyticus*, pouco é sabido dos fatores de virulência e característivas epidemiológicas dessas cepas. Avaliar o perfil uropatogênico desses isolados propicia um melhor entendimento da relação parasita-hospedeiro de *S. saprophyticus*.

OBJETIVOS

Objetivo Geral:

Caracterizar os isolados de *Staphylococcus* do grupo *saprophyticus* e seus potenciais fatores de virulência.

Objetivos Específicos:

Avaliar o desempenho dos métodos automatizados Vitek 2 e Vitek MS (MALDI-TOF) quanto a acurácia na identificação de isolados clínicos e cepas-referência de cocos Gram-positivos e, especificamente, de *Staphylococcus* do grupo *saprophyticus*.

Caracterizar fenotipicamente isolados de *Staphylococcus saprophyticus* pelo método convencional de identificação descrito na literatura.

Avaliar o perfil de suscetibilidade aos antimicrobianos de isolados de *Staphylococcus saprophyticus*.

Avaliar os fatores de virulência associados à adesão (formação de biofilme e aderência à linhagem uro-epitelial T-24) de isolados clínicos de *Staphylococcus saprophyticus*.

Avaliar a prevalência de genes associados à adesão (*aas, uafA, sdrI e ssp*) de *Staphylococcus saprophyticus* ao uro-epitélio e as proteínas da matriz extracelular do hospedeiro.

MANUSCRITO I - Submetido para a revista *Diagnostic Microbiology and Infectious Disease* em 22 de Novembro de 2012

Performance of VITEK 2 Compact System (Software Version 5.03) in the Bacterial Identification and Antimicrobial Susceptibility Test: Evaluation Study with Clinical and Reference Strains of Gram-positive Cocci.

Running title: Gram-positive Cocci Identification by VITEK 2

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Abstract

The aim of the study was evaluate the performance of VITEK 2 in the identification and antimicrobial susceptibility in bacterial Gram-positive cocci. One hundred four isolates was analyzed, 29 reference strains and 75 clinical samples identified by conventional methods, and the antimicrobial susceptibility test (AST) was evaluated for oxacilin and vancomicin. The VITEK 2 was performed according to manufacture instructions. The system correctly identified 77.9% and 97.1% of the isolates to the species and genus level, respectively (77.8% for streptococci, 78% for staphylococci and 77.8% for enterococci). Moreover, multiple identification was provided in 11 of 23 not identified strains, and 7 reference strains were not identified. The rate of strains with agreement to conventional AST profile was 81.8%. The VITEK 2 correctly identified strains commonly isolated, however, method limitations can result in ambiguous findings and the inability to identify uncommon microorganisms, requiring additional tests to identification at specie-level.

Introduction

The Gram-positive cocci are widely distributed as part of the normal flora in humans, however, are recognized as major human pathogens, causing a large variety of infections worldwide. These microorganisms are frequently isolated from bloodstream infection, skin and soft tissue infections, sepsis, urinary tract infections and lower respiratory tract infections (Johannes, 2008; Empinotti et al, 2012; Landrum et al, 2012; Ludwig et al, 2012; Neuzillet et al, 2012).

The high number of enterococci, staphylococci and streptococci species involved in infectious disease can be identified by biochemical conventional methodology, however, this method is relatively laborious for routine laboratories, since this technique are composed of large number of tests, are complicated to use and requires prolonged incubation time for identification at the species level. Although simple schemes, showing good levels of accuracy, has been proposed as identification alternative, these methods identify few species and still use at least 72h of incubation, it is not feasible in routine microbiology laboratory. (Kloos and Schleifer, 1975; Manero and Blanch, 1999, De Paulis et al, 2003; Cunha et al, 2004; Iorio et al, 2007)

The automated bacterial identification in the clinical laboratory provides rapid and reliable diagnostic for the most pathogens involved in the infectious diseases (Pumarola, 2010). Moreover, the high degree of automation may also improve accuracy to bacterial identification (Funke et al, 1998). Previous studies have demonstrated the satisfactory performances of the automated methodologies, allowing their use in routine practice with a highly acceptable level of identification accuracy, besides enabling interpretation of antimicrobial susceptibility tests for correct treatment of patients (Wallet et al, 2005; Mory et al, 2009).

However, evaluation performance studies of automated methodologies are essential for checking the accuracy of the systems in the discrimination of different bacterial isolates that may be involved in infectious diseases. Studies show that the agreement rates of the automated systems are dependent of microorganisms, since phenotypic characteristics may be variable or poor discriminatory, and depend on the expression of metabolic activities and/or morphological features, where these methods may fail to discriminate closely related species. Furthermore, it is a reality the onset of infectious diseases by bacteria not previously described in certain diseases, as well as a possibility that the automated system can report an erroneous identification of the pathogen isolated from the patient (Layer et al, 2006; Gupta et al, 2011).

In recent meta-analysis study, species identification of commonly isolated Gram-positive cocci, as *Staphylococcus aureus*, were significantly more accurate in comparison to coagulase-negative *Staphylococcus* (CoNS) by VITEK 2 system (bioMérieux, Marcy l'Etoile, France) (Chatzigeorgiou et al, 2011). This result is in accordance with previous study, where most of the discrepant bacterial identification involved the less frequently isolated species, requiring further improvement in order to provide reliable results for characterization of these isolates (d`Azevedo et al, 2009).

The aim of the present study was evaluate the performance of VITEK 2 automated system software version 5.03 in the bacterial identification and antimicrobial susceptibility with Gram-positive cocci isolates recovered from clinical samples and reference strains.

Material and Methods

Bacterial Strains

The study was performed at Laboratório de Cocos Gram-Positivos da UFCSPA (LCGP) and Laboratorio Qualità, RS, Brazil. The isolates included in present study were selected from strain collection belonging LCGP, and all were stored at -20 C in skin milk (Becton Dickinson S.A., USA). A total of 104 isolates of Gram-positive cocci was analyzed, 29 reference strain selected from the American Type Culture Collection (ATCC) – *Streptococcus agalactiae* ATCC 13813, *Streptococcus galollyticus* ATCC 9809, *Streptococcus equi* subsp. *equi* ATCC 9528, *Streptococcus parasanguinis* ATCC 903, *Streptococcus oralis* ATCC 10557, *Streptococcus salivarius* ATCC 7073, *Streptococcus pneumoniae* ATCC 33400, *Streptococcus mutans* ATCC 25175, *Streptococcus* sp. ATCC 15300, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* SS 1273 and *Enterococcus faecium* SS 1274 (reference strains from Streptococcus Laboratory – Center for Disease and Prevention Control, CDC), *Staphylococcus epidermidis* ATCC 35984, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 13150, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 12600, *Staphylococcus caprae* ATCC 35538, *Staphylococcus intermedius* ATCC 29663, *Staphylococcus saprophyticus* ATCC 15305, *Staphylococcus hominis* ATCC 27844, *Staphylococcus haemolyticus* ATCC 29970, *Staphylococcus xylosus* ATCC 29971, *Staphylococcus warneri* ATCC 10209, *Staphylococcus cohnii* subsp. *cohnii* ATCC 29974, *Staphylococcus capitis* subsp. *capitis* ATCC 27840 – and 75 clinical isolated obtained from different patients – *Staphylococcus* coagulase-negative (n=36), *Enterococcus* sp (n=33) and *Staphylococcus aureus* (n=6).

Phenotypic Reference Method for Gram-positive Cocci Identification

The clinical isolates tested were identified to the species level by conventional methods described by Bannerman and Peacock (2007) and Antunes et al (2008) for staphylococci, and Teixeira et al (2007) for enterococci. For identification of staphylococci, the following characteristics were tested: catalase, colony morphology and pigmentation, Gram stain, hemolysis, susceptibility to novobiocin, polymyxin B, fosfomycin and deferoxamine, enzyme activity of arginine arylamidase, ornithine decarboxylase and urease, and acid production from trehalose, mannitol, mannose, xylose, cellobiose, arabinose, maltose, lactose, sucrose and raffinose. For the enterococcal isolates, the following phenotypic characteristics were evaluated: catalase, colony morphology and pigmentation, hydrolysis of esculin in presence of 40% bile, growth in 6.5% NaCl, motility, and acid production from mannitol, sorbose, arginine, arabinose, sorbitol, raffinose, sucrose, pyruvate and methyl-glucopyranoside.

Antimicrobial Susceptibility Testing (AST)

For the staphylococcal and enterococcal isolates, susceptibility to oxacilin and vancomicin was evaluated by Broth Microdilution (BMD) and disk-diffusion (DD) reference methods according CLSI documents M7-A6 (2003) and M100-S21 (2011). The minimal inhibitory concentration (MIC) was defined, and categorization as susceptible, intermediate or resistant was in accordance with the CLSI (2011) recommendations.

Automated identification by VITEK 2

The VITEK 2 test method (software version 5.03) was evaluated using GP-ID card for bacterial isolates identification according to manufacture instructions. The isolates were

cultured on TSA (Trypticase Soy Agar) with 5% defibrinated sheep blood (bioMe´rieux, Marcy L'E`toile, France) for 24h at 35±2 °C. The colonies was suspended in sterile saline (0.45%) and adjusted to 0.5 at 0.63 McFarland turbidity using DensiChek Densitometer (bioMe´rieux) and the tubes were placed in a cassette on the VITEK 2 Smart Carrier Station. After the GP card incubation, the results were analyzed as to accuracy of bacterial identification.

Automated AST by VITEK 2

The AST was performed according the manufacturer's instruction. Bacterial suspension was prepared in 0.45% saline to a density of 0.5 – 0.63 using DensiChek Densitometer. The AST-cards were inoculated with an appropriated dilution and loaded into the VITEK2 instrument for incubation and reading. The Minimal Inhibitory Concentration (MIC) analysis and interpretations were evaluated using the VITEK 2 Advanced Expert System (AES).

Data analysis

The comparative analysis of bacterial identification by VITEK 2 and the conventional method was performed, and the accuracy (percentage of match identification) was characterized. Descriptive measures were conducted to evaluate the identification performance of the automated method at the genus and species levels. The results that were not identical with those obtained by the conventional phenotypic method were classified as: i) misidentification (discrepant identification in comparison with conventional methodology) or ii) low-level discrimination (identification result whereby two or more bacterial species were

reported by VITEK 2 system). ANOVA analysis was performed to evaluate the statistical significance among the bacterial group for time to final identification. If a significant F value was found, Student-Newman-Keuls post hoc test was performed to identify the difference among the groups.

The AST results evaluated with the automated system (VITEK 2) were compared to those obtained with reference method and analyzed as to agreement rates. The type of error was evaluated by following criteria: i) Very Major Error (VME) defined when an resistant bacterial isolate appeared susceptible by the VITEK 2; ii) Major Error (ME) defined when susceptible isolate showed the resistant profile by the VITEK 2; or iii) Minor Error (MiE) occurred with microorganisms for which AST reference indicated intermediate resistance and susceptibility or resistance by automated AST test.

Results

The VITEK 2 system correctly identified 81 (77.9%) and 101 (97.1%) of 104 bacterial isolates to the species and genus level, respectively. Genus identification was correct among 96.7% of *Staphylococcus* spp, 92.1% of *Enterococcus* sp and 88.9% of *Streptococcus* spp strains. The performance of VITEK 2 system to the correct identification at species level reached 77.8% for streptococci, 78% for staphylococci and 77.8% for enterococcal isolates. No significant difference was detected among the genera of the study for time to final identification ($p=0.71$), however, *S. aureus* was faster identified than others staphylococcal isolates ($p<0.05$) (Table 1).

The VITEK 2 automated method showed accuracy at the species level identification for commonly isolated Gram-positive cocci. All the clinical isolates *S. aureus* and *E. faecalis* were correctly identified, as well as bacterial strains as *S. capitis*, *S. caprae*, *S. cohnii*, *S. lugdunensis*, *E. hirae* and *Leuconostoc* sp (Table 2). Bacterial isolates were misidentified or

showed low-level discrimination in clinical samples of *S. epidermidis* (25%), *S. haemolyticus* (66.7%), *S. hominis* (16.7%), *S. saprophyticus* and *S. warneri* (both 20%). In the *Enterococcus* strains, *E. avium* and *E. durans* were misidentified as others enterococcal isolates, and the discordant results were found 50% of *E. casseliflavus*, 75% of *E. faecium* and 25% of *E. gallinarum* isolates. Among the 29 reference strains, a definitive species identification was provided for 22 (75.9%) by the automated system (Table 2).

The incorrect identification or low-level discrimination results are listed in Table 3. There were 23 strains for which the VITEK 2 could not provide a definitive specie identification. Multiple identification was provided by the automated method in 11 of 23 not identified bacterial isolates. Interestingly, 7 were reference strains of 23 bacterial isolates that showed not agreement identification between the conventional phenotypic method and the VITEK2 system. These, six was misidentified and one reference strain showed low-level discrimination (Table 3).

Low-level discrimination at two or more bacterial species has been found in the following isolates: *S. epidermidis* (*S. hominis* subs / *S. epidermidis*), *S. haemolyticus* (*S. warneri* / *S. hominis* subs *hominis* / *S. haemolyticus* / *Aerococcus viridans*), *S. hominis* subs *hominis* (*S. auricularis* / *S. hominis* subs *hominis*), *S. warneri* (*S. hominis* subs *hominis* / *S. warneri*), *E. casseliflavus* (*E. casseliflavus* / *E. gallinarum*), *E. faecium* (*E. durans* / *E. faecium*) and *E. gallinarum* (*E. faecium* / *E. gallinarum*). The percent of probability for the each isolate is described in the Table 3.

Minimal Inhibitory Concentration (MIC) of oxacilin and vancomycin generated by the automated system were compared as to error type and showed in the Table 4. The number of bacterial isolates with agreement to conventional antimicrobial susceptibility profile was 45 of 55 (81.8%), with agreement rate of 88.4% for staphylococci and 77.3% for enterococci samples. Three very major error (VME) and 2 major error (ME) were found in

staphylococcal isolates, whereas 1 and 4 enterococci showed VME and ME, respectively.

Discussion

The automated VITEK 2 system was able to identify, at the species level, commonly isolated strains of staphylococci and enterococci, as *S. aureus* and *E. faecalis*, respectively, which all the isolates was correctly identified, and reference strains of streptococci. These results were in agreement with those reported by Chatzigeorgiou et al (2010), that evaluated the performance of VITEK2 system in comparison with other automated system (Chatzigeorgiou et al, 2010). However, in the present study, VITEK 2 version 5.03 software was not able to correctly identified all the *S. epidermidis* isolates, showing match at the specie level of 71.4% (one reference strains with low-level identifications between *S. hominis* and *S. epidermidis* species, and 1 clinical isolate misidentified as *S. hominis*). Interestingly, although Kim et al (2008) has been found an agreement rate slightly higher than our study (88.7% to *S. epidermidis* identification), their analysis of the isolates resulted in multiple identifications as *S. hominis* and *S. epidermidis* (Kim et al 2008). Similarly, a previous study reported misidentification and low-level discrimination between these coagulase-negative staphylococci species (Spanu et al, 2003).

Although, in recent comparative study with five methodologies for CoNS identification, Loonen et al indicate better performance of VITEK 2 for these isolates (92.3%), the bacterial species that showed incorrect identification were *S. capitis*, *S. epidermidis*, *S. haemolyticus* and *S. hominis* (Loonen et al, 2012). The correct identification of these species is relevant to the clinical laboratory, since *S. epidermidis* is recognized as an opportunistic pathogen , mainly for infections that involve biofilm formation (Otto, 2012) and *S. hominis* is opportunistic microorganism that may cause infection in patients with

abnormality of immune system (Jiang et al, 2012). In our study, bacterial isolates were misidentified or showed low-level discrimination between *S. epidermidis* and *S. hominis* (see Table 3). Although these species show similar phenotypic profile by automated system, fosfomycin disks can be used as feasible test to differentiate this strains (*S. epidermidis* is susceptible to fosfomycin and *S. hominis* is resistant) (Antunes et al, 2008), which characterizes a minor error in the bacterial identification.

S. haemolyticus is frequently isolated from blood cultures and has a tendency to develop resistance to multiple antimicrobial drugs (Barros et al, 2012), likewise *S. warneri*, another CoNS which can cause catheter-related bacteremia, native and prosthetic valve endocarditis (Arslan et al, 2011). Two isolates of this bacterial specie were misclassified as *S. warneri* by the VITEK 2 system. Moreover, some *S. haemolyticus* isolates were misidentified or showed low-level discrimination among *S. hominis* and *Aerococcus viridans* isolates (see Table 3). According to a previous study, similar results were found by Delmas et al (2008). However, these species can be easily discriminated with a fast and easily preprocessing of the samples, as catalase test (*Aerococcus* species are negative and *Staphylococcus* shows a positive reaction) and evaluation the activity of pyrrolidonyl arylamidase (PYR) test (performed by VITEK 2 system) and desferroxamine susceptibility (only *S. haemolyticus* is PYR positive, while *S. warneri* and *S. hominis* are PYR negative but *S. warneri* is desferroxamine resistant) (Iorio et al, 2007). These characteristics of the biochemical profile are enough to differentiate these staphylococcal strains at the species level.

Two *S. saprophyticus* were misidentified as *S. warneri* and *S. cohnii* subs. *urealyticus* by VITEK 2 automated system. According to the biochemical profile of these isolates, major error in the bacterial identification can be evaluated by novobiocin susceptibility, since *S. saprophyticus* and *S. cohnii* are resistant, while *S. warneri* is susceptible (Iorio et al, 2007; Hwang et al, 2011). Moreover, *S. saprophyticus* and *S. cohnii* subs. *urealyticus* have similar

phenotypic profile, showing low discriminatory power in bacterial identification (both species are urease positive and novobiocin resistant). The clinical laboratory should be able to differentiate these strains, since *S. cohnii* subs. *urealyticus* is recognized as pathogen in infectious diseases as endocarditis, septicaemia and urinary tract infections, however, previous studies have reported the difficulty in routinely identifying this microorganism (Basaglia et al, 2003; d'Azevedo et al, 2007; d'Azevedo et al, 2008). These two related isolates can be sufficiently discriminated by mannose acid production, providing correct identification at the species-level (Bannerman et al, 2007; Iorio et al, 2007).

In the present study, the agreement identification rate of enterococci isolates was similar a previous study, and all the *E. faecalis* were correctly identified by VITEK 2 system (d'Azevedo et al, 2009). However, 3 of 5 *E. faecium* strains were misclassified or showed low-level discrimination as *E. durans*, *E. gallinarum* and *E. faecalis*. The bacterial identification of *Enterococcus* isolates is important because the genus includes some of the most important multidrug-resistant organisms in healthcare-associated infection, and these isolates usually affect patients who are debilitated by other, concurrent illnesses and undergoing prolonged hospitalization, including *E. faecium* strains, a pathogen with the ability to succeed in the hospital environment (Arias and Murray, 2012). These strains have similar phenotypic characteristics, with exception of methyl-alfa-D-glucopyranoside (MGP) acid production and motility (*E. gallinarum* is MGP and motility positive, while *E. faecium* is not), and arabinose and mannitol acid production (*E. faecalis* is negative to arabinose and mannitol positive, but *E. faecium* shows both positive reactions, while *E. durans* shows both negatives) (Manero and Blanch, 1999). The fewer additional tests allow the correct identification at the specie-level of these related enterococci strains, similarly to *E. gallinarum* and *E. cassiliflavus* species, both motile microorganisms but *E. casseliflavus* possesses colony pigmentation (Manero and Blanch, 1999; Teixeira et al, 2007).

Our data indicated that the VITEK 2 system provided inaccurate susceptibility test results for oxacillin and vancomycin, since the agreement with the reference method was well below, and the error rates (mainly VME and ME) were higher than compared with other studies (Garcia-Garrote et al, 2000; Ligozzi et al, 2002; Nonhoff et al, 2005; Gherardi et al, 2012). Although our study has been evaluated the antimicrobial susceptibility to commonly isolated strains, as *S. aureus* and *E. faecalis*, most of isolates were microorganisms that belong CoNS and enterococci non-faecalis strains. A previous meta-analysis study reports that the discordant results in the bacterial identification could be explained by these bacterial isolates that show slow metabolic rates, leading to ambiguous reactions within the short incubation times used by automated instruments (Chatzigeorgiou et al, 2011). These characteristics of the sample could interfere with AST automated method, since staphylococci and enterococci isolates showed very major errors (resistant strains were categorized as susceptible microorganisms).

In conclusion, VITEK 2 Compact system software version 5.03 correctly identified commonly Gram-positive cocci isolates, however, method limitations can result in ambiguous findings and the inability to identify uncommon microorganisms. Therefore, additional phenotypic tests can be necessary to identify some strains at species-level, as well as critical inquiry of AST results reported by the automated method, since discrepancies in the antimicrobial susceptibilities might occur in uncommon isolated pathogens.

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TABLES

Table 1: Descriptive measures from automated system VITEK 2 Compact

Bacterial isolates		Identification (%)		Time to identification (h)[a]
		genus level	species level	
Staphylococcus spp.		96.7	78	6.03 (1.20)[c]
	S. aureus	100	100	4.82 (0.68)[d]
	S. epidermidis	100	71.4	6.18 (1.40)[d]
	others CoNS[b]	95.2	71.4	6.31 (1.14)[d]
Enterococcus sp.		92.1	77.8	5.84 (1.12)[c]
	E. faecalis	100	100	5.53 (0.79)[e]
	E. faecium	100	40	5.60 (1.34)[e]
	Others	91.7	58.3	6.46 (1.30)[e]
Streptococcus spp.		88.9	77.8	5.81 (1.49)[c]
Micrococacceae		96.7	76.7	6.03 (1.21)[f]
Streptococcaceae		95.5	77.8	5.84 (1.18)[f]

[a] mean (SD - Standard deviation)
[b] Coagulase-negative Staphylococcus non-epidermidis
[c] p=0.71
[d] p<0.05
[e] p=0.06
[f] p=0.41

Table 2: Performance of VITEK 2 system to bacterial isolates of the study.

Species or family (no.)	No. (%) bacterial isolates that were:	
	Correctly identified	Misidentified or low discrimination
Staphylococcus spp. (42)	34 (81.0)	8 (19.0)
S. aureus (6)	6 (100)	0 (0)
S. capitis (4)	4 (100)	0 (0)
S. caprae (1)	1 (100)	0 (0)
S. cohnii (4)	4 (100)	0 (0)
S. cohnii subs. cohnii (2)	2 (100)	0 (0)
S. cohnii subs. urealyticus (2)	2 (100)	0 (0)

	S. epidermidis (4)	3 (75)	1 (25)
	S. haemolyticus (6)	2 (33.3)	4 (66.7)
	S. hominis (6)	5 (83.3)	1 (16.7)
	S. hominis subs. hominis (3)	2 (66.7)	1 (33.3)
	S. hominis subs.novobiosepticus (3)	3 (100)	0 (0)
	S. lugdunensis (1)	1 (100)	0 (0)
	S. saprophyticus (5)	4 (80)	1 (20)
	S. warneri (5)	4 (80)	1 (20)
Enterococcus sp. and related genus (33)		25 (75.7)	8 (24.3)
	E. avium (1)	0 (0)	1 (100)
	E. casseliflavus (2)	1 (50)	1 (50)
	E. durans (2)	0 (0)	2 (100)
	E. faecalis (17)	17 (100)	0 (0)
	E. faecium (4)	1 (25)	3 (75)
	E. gallinarum (4)	3 (75)	1 (25)
	E. hirae (2)	2 (100)	0 (0)
	Leuconostoc spp. (1)	1 (100)	0 (0)
Reference strains (29)		22 (75.9)	7 (24.1)
	Enterococcus faecalis (2)	2 (100)	0 (0)
	Enterococcus faecium (1)	1 (100)	0 (0)
	Staphylococcus aureus (5)	5 (100)	0 (0)
	Staphylococcus capitis subs capitis (1)	1 (100)	0 (0)
	Staphylococcus caprae (1)	0 (0)	1 (100)
	Staphylococcus cohnii subs. cohnii (1)	1 (100)	0 (0)
	Staphylococcus epidermidis (3)	2 (66.7)	1 (33.3)
	Staphylococcus haemolyticus (1)	0 (0)	1 (100)
	Staphylococcus hominis (1)	1 (100)	0 (0)
	Staphylococcus intermedius (1)	0 (0)	1 (100)
	Staphylococcus saprophyticus (1)	0 (0)	1 (100)
	Staphylococcus warneri (1)	1 (100)	0 (0)
	Staphylococcus xylosus (1)	1 (100)	0 (0)
	Streptococcus agalactiae (1)	1 (100)	0 (0)
	Streptococcus equi subs. equi (1)	0 (0)	1 (100)
	Streptococcus gallolyticus (1)	1 (100)	0 (0)
	Streptococcus mutans (1)	1 (100)	0 (0)
	Streptococcus oralis (1)	1 (100)	0 (0)
	Streptococcus parasanguinis (1)	1 (100)	0 (0)
	Streptococcus pneumoniae (1)	1 (0)	0 (0)
	Streptococcus salivarius (1)	0 (0)	1 (100)
	Streptococcus sp.(1)	1 (100)	0 (0)
Micrococacceae (59)		46 (78.0)	13 (22.0)

Streptococacceae (45)	35 (77.8)	10 (22.2)

Table 3: Bacterial species reported by VITEK 2 System that were misidentified or showed low discrimination

Reference bacterial identification (no. isolates)	VITEK 2 identification (% probability)
Staphylococcus caprae ATCC 35538 (1)	*S. cohnii subs. urealyticus* (99%)
Staphylococcus epidermidis (1)	*S. hominis subs. hominis* (51%)
	S. hominis subs. novobiosepticus (49%)
Staphylococcus epidermidis ATCC 12228 (1)	*S. epidermidis* (50%)
	S. hominis subs. hominis (50%)
Staphylococcus haemolyticus (1)	*S. warneri* (50%)
	S. hominis subs. hominis (50%)
Staphylococcus haemolyticus (2)	*Aerococcus viridans* (50%)
	S. hominis subs. hominis (50%)
Staphylococcus haemolyticus (1)	*Aerococcus viridans* (33%)
	S. haemolyticus (33%)
	S. hominis subs. hominis (33%)
Staphylococcus haemolyticus ATCC 29970 (1)	*S. warneri* (95%)
Staphylococcus hominis subs. hominis (1)	*S. auricularis* (50%)
	S. hominis subs. hominis (50%)
Staphylococcus intermedius ATCC 29663 (1)	*S. chromogenes* (89%)
Staphylococcus saprophyticus (1)	*S. warneri* (93%)
Staphylococcus saprophyticus ATCC 15305 (1)	*S. cohnii subs. urealyticus* (89%)
Staphylococcus warneri (1)	*S. hominis subs. hominis* (50%)
	S. warneri (50%)
Enterococcus avium (1)	*E. faecalis* (99%)
Enterococcus casseliflavus (1)	*E. casseliflavus* (50%)
	E. gallinarum (50%)
Enterococcus durans (1)	*E. hirae* (99%)
Enterococcus durans (1)	*Pediococcus acidilactici* (91%)
Enterococcus faecium (1)	*E. durans* (50%)
	E. faecium (50%)
Enterococcus faecium (1)	*E. gallinarum* (99%)

Enterococcus faecium (1)	*E. faecalis* (98%)
Enterococcus gallinarum (1)	*E. faecium* (51%)
	E. gallinarum (49%)
Streptococcus equi subs. equi ATCC 9528 (1)	*E. faecalis* (98%)
Streptococcus salivarius ATCC 7073 (1)	*S. equinus* (98%)

Table 4 Antimicrobial susceptibility test agreements and type of error among staphylococci and enterococci isolates*

Isolates (no.)	No. and (%) of agreements	No. and (%) of errors	Type of error (no.)[c]		
			VME	ME	MiE
Staphylococci (33)[a]	28 (84.8)	5 (15.2)	3	2	0
Enterococci (22)[b]	17 (77.3)	5 (22.7)	1	4	0

[a] Susceptibility of oxacilin

[b] Susceptibility of vancomicin

[c] VME (Very Major Error); ME (Major Error); MiE (Minor Error)

* Reference strains of streptococci not tested.

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

Running title: MALDI-TOF performance for Gram-positive cocci identification

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Abstract

Until recently, Gram-positive cocci identification has mainly relied on conventional and time-consuming phenotypic methods. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid alternative for bacterial identification and our study aimed to compare the performance of this methodology with the golden standard method for Gram-positive cocci identification. *Staphylococcus* spp (n=386), *Enterococcus* sp (n=46), *Streptococcus* spp (n=18) clinical isolates and reference strains were studied. MALDI-TOF MS methodology yielded agreement identification of 440 strains out of 450 (97.8%) identified by conventional phenotypic method. The species with more disagreements on identification was *S. epidermidis* (n=5). Some *S. haemolyticus* isolates displayed two distinct genus identification at the first acquisition by MALDI-TOF MS. Two *E. gallinarum* were misidentified as *E. faecium* and one streptococci isolate was erroneously identified by MALDI-TOF (*S. gordonii* as *S. mitis/oralis*). Our data suggest that MALDI-TOF MS is fast and reliable, and can be implemented in a clinical microbiology laboratory setting, mostly due to accuracy for Gram-positive cocci identification.

Introduction

Gram-positive cocci identification has been always a challenge for microbiology laboratories around the world. The emergence of *Staphylococcus* coagulase-negative (SCoN) as pathogens and antimicrobial resistance reservoirs had required the application of reliable identification methods, either for establishing epidemiological trends as for confirming treatment failures in order to clearly understand pathogenicity mechanisms.[1, 2, 3] Likewise, *Enterococcus* sp became an important pathogen due to their increasing role as opportunistic agents and their special ability to acquire resistance to antimicrobial drugs, including vancomycin.[4] In addition, *Streptococcus* spp are an heterogeneous group that have been linked to infective endocarditis, invasive pyogenic infections, septic arthritis, subacute bacterial endocarditis, pharyngitis, impetigo, and pneumococal pneumonia, the major cause of worldwide mortality for this genus.[5, 6, 7, 8, 9]

Until recently, bacterial identification has mainly relied on conventional and time-SCoNuming phenotypic methods, commercial or automated systems that often do not distinguish the variable expression of some staphylococcal phenotypic characteristics, and gene sequencing identification techniques.[1, 3, 10] Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid alternative for bacterial identification, based on the ionization of co-crystallized sample material (microbial cells protein composition) by short laser pulses.[11] The ions are accelerated and their time of flight is measured in a vacuum flight tube, yielding a bacterial mass spectrum.[12] Paradox between the fact that conventional methodology could discriminate among small phenotypic differences in SCoN and automated systems could not may be solved or attenuated by this alternative instrument, since its principle is based on specific protein profiles, closely similar to a molecular identification method as 16S rDNA sequencing.[13]

The aim of the present study was to compare the MALDI-TOF MS methodology and

conventional phenotypic methods to identify Gram-positive cocci isolates recovered from clinical specimens.

Material and Methods

Bacterial strains

Staphylococcus spp (n=386), *Enterococcus* sp (n=46) and *Streptococcus* spp (n=18) recovered from different clinical samples between 2002 and 2010, as well as reference strains, were studied. These isolates were obtained from patients attending at three hospital facilities in Porto Alegre, south Brazil and by American Type Culture Collection (ATCC). The isolates were subcultured on 5% blood sheep agar (bioMeriéux, Marcy I'Etoile, France) and incubated at 35°C for 24h. Then, they were submitted either to phenotypic methods as to analysis by MALDI-TOF MS methodology.

Conventional phenotypic method

All the clinical isolates were submitted to conventional phenotypic tests by the methodology proposed by Bannerman and Peacock[14] and Antunes[15] for staphylococci, Teixeira[16] for enterococci, and Spellerberg and Brandt[17], Beck, Frodl and Funke[18] for streptococci *bovis* group. For identification of staphylococci, the following characteristics were tested: catalase, colony morphology and pigmentation, Gram stain, hemolysis, susceptibility to novobiocin (5μg), polymyxin B (300U), fosfomycin (200μg) and deferoxamine (100μg), enzyme activity of arginine arylamidase, ornithine decarboxylase and urease, and acid production from trehalose, mannitol, mannose, xylose, cellobiose, arabinose, maltose, lactose, sucrose and raffinose. For the enterococcal isolates, the following phenotypic characteristics were evaluated: catalase, colony morphology and pigmentation, hydrolysis of esculin in presence of 40% bile, growth in 6.5% NaCl, motility, and acid

production from mannitol, sorbose, arginine, arabinose, sorbitol, raffinose, sucrose, pyruvate and methil-alfa-D-glucopyranoside. The species belonging to the *Streptococcus bovis* group analyzed in the present study (*S. gallolyticus* and *S. infantarius*) were tested for Lancefield group D reaction (Oxoid, Hampshire, United Kingdom), hydrolysis of esculin in presence of 40% bile, growth in 6.5% NaCl and acid production from mannitol, methil-alfa-D-glucopyranoside and trehalose. All tests results were obtained after incubations of 24h, 48h and 72h at 35°C.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

The sample preparation for mass spectrometry was carried out as follow. Each steel slide contained three acquisitions groups, and each acquisition group contained 16 spots, being able to perform 48 different isolates. An amount of a freshly grown 24-hour-old colony was placed directly onto a steel target sample spot in a thin film. This film was then overlaid with 1µl of a saturate matrix solution of α-cyano-4-hydroxycinnamic acid and dried at room temperature. The slide was then inserted into the MALDI-TOF MS instrument (bioMeriéux, Marcy I'Etoile, France). The mass spectra generated were analyzed and compared with a reference spectra database.

Data analysis

Descriptive measures were evaluated from MALDI-TOF results. Identification agreement between conventional phenotypic method and MALDI-TOF were computed for the accuracy determination of the method. Misidentification rates were evaluated by type error as major error (disagreement at genus level) and minor error (disagreement at species level).

Identification criteria used in our study were based on the reliability provide by the instrument, which was based in probability: one microorganism with ≥90% indicated conclusive identification if same genus, two microorganisms with ≥90% indicated inconclusive identification if same genus, one or two microorganisms with ≥90% indicated unreliable identification if different genus. When the instrument was not able to provide any identification, it was considered technique limitation.

Result

The accuracy of MALDI-TOF MS for bacterial identification and the misidentified isolates were provided, in detail, in the Table1 and Table 2, respectively. All *Staphylococcus aureus* isolates were correctly identified by MALDI-TOF MS methodology. The method accuracy for SCoN isolates was 98% and all *S. haemolyticus* and *S. hominis* species were correctly identified. *S. epidermidis* identification matched at the species level in 89 of 94 isolates tested (identification agreement of 94.7%). Five strains were discordant, all presenting minor error: two *S. epidermidis* were erroneously identified as *S. warneri*, and strains misidentified as *S. hominis* subs. *hominis, S. intermedius* and *S. saprophyticus*. MALDI-TOF MS failed to identify three *S. epidermidis* isolates in the first acquisition set; however, all were correctly identified in further analysis. The new methodology yielded correct identification at species level for *all S. haemolyticus* isolates (n=106), although ten strains had to be tested again. Interestingly, four isolates had the wrong genus identification (major error) in the first acquisition by MALDI-TOF MS, with three identifications SCoN consisting of *Corynebacterium aurimucosum* and one of *Enterococcus faecalis*. Moreover, six isolates could not be identified in the first analysis, only being identified in the further acquisition.

Among *S. saprophyticus* isolates, identification matched at the species level was 98.9%; one strain could not be identified. Similarly, all the twenty-six strains of *S. hominis* were correctly identified in the species-level (100%), with one isolate not identified in the first accquisition. Others SCoN analyzed in this study (*S. capitis, S. caprae, S. cohnii, S. intermedius, S. lugdunensis, S. sciuri* and *S. xylosus*) showed identification agreement at the species-level between the conventional phenotypic method and MALDI-TOF MS. Discordant results was yielded by one *S. warneri* isolate, which produced two bacterial mass spectrum with identical probability (*Staphylococcus warneri* 99.9% / *Prevotella buccalis* 99.9%).

For enterococcal isolates, identification agreement was 95.7%. All *E. faecalis, E. faecium, E. casseliflavus* and *E. hirae* isolates were correctly identified by MALDI-TOF methodology. However, correct genus but incorrect species identification was provided for two of six *E. gallinarum* strains. These isolates were misidentified as *E. faecium*, therefore, classified as minor error (Table 2).

A group of miscellaneous *Streptococcus* species was analyzed by MALDI-TOF MS methodology. A total of 17 out of 18 streptococci isolates were identified at species level, with agreement identification rate of 94.4%. One *S. gordonii* strain was misidentified as *Streptococcus mitis/Streptococcus oralis* group (minor error) and all *S. gallolyticus* isolates (n=7) matched exactly at subspecie-level by the methodology of study.

Discussion

An identification technique for clinically relevant bacteria has to be fast, accurate and reliable. However, conventional phenotypic methods for Gram-positive cocci are relatively problematic for use in routine laboratories, with biochemical tests that require more than 72 hours for species-level identification.[19] Currently, automated identification systems and

miniaturized methods are commonly used in laboratories for rapid identification of microorganisms, but the accuracy for some Gram-positive cocci has been found to be lower than the one of the reference method. It could be probably due to ambiguous reactions, phenotypic variation, atypical biochemical characteristics and slow growth rates, leading to misidentification within the short incubation times used by automated instrument.[19, 20]

The MALDI-TOF MS methodology, when implemented in the clinical laboratories, has been reported to be efficient, cost-effective and rapid in microorganisms identification. It enabled wide bacterial species range identification and, under routine laboratory conditions, showed a high accurate identification rate at the species level.[21, 22]

In this study, MALDI-TOF methodology yielded agreement identification of 440 (97.8%) of 450 Gram-positive cocci isolates identified by conventional phenotypic method. The better performance of the new methodology was to staphylococci, followed for enterococci and streptococci isolates. Among SCoN, correctly identification rate at the species level was 98% (336/343), with 6 isolates misidentified and 1 not identified. These results were like previous reports, which included SCoN clinical isolates.[23] Dubois et al and Carpaij et al, in recent studies, demonstrated that MALDI-TOF, compared to molecular identification of clinical and reference strains, is an accurate method for important SCoN species determination (accuracy of 99.3% and 100%, respectively), a method that could be implemented for identification of this Gram-positive cocci group in routine clinical microbiology.[24, 25]

The misidentified *S. epidermidis* isolates (identified as *S. warneri, S. hominis* subs. *hominis, S. intermedius* and *S. saprophyticus*) could be discriminated by conventional biochemical tests, as bound coagulase (positive results for *S. intermedius*) novobiocin susceptibility (among these species, only *S. saprophyticus* is resistant) and desferrioxamine susceptibility (*S. epidermidis* and *S. hominis* are susceptible, but *S. warneri* is not).[19] Although

S. epidermidis and *S. hominis* species show similar phenotypic profile, fosfomycin disks can be used as feasible test to differentiate this strains (*S. epidermidis* is susceptible to fosfomycin and *S. hominis* is resistant).[15]

The MALDI-TOF MS system identified one *S. warneri* isolate with mass spectrum of two unrelated bacteria - *Staphylococcus warneri* 99.9% / *Prevotella buccalis* 99.9%. *Prevotella buccalis* is an anaerobic Gram-negative bacilli isolated from the human oral microbiota.[26] Phenotypically, both microorganisms can be differentiated by prior samples processing, without any complementary tests.

Some *S. haemolyticus* isolates displayed two distinct genus identification at the first acquisition by MALDI-TOF MS. *S. haemolyticus* isolates were identified incorrectly as *Corynebacterium aurimucosum*, microorganism that belongs to the normal human flora; some species are common contaminants and occasional causes of prosthetic joint infection.[27, 28] Similarly, the methodology inaccurately identified one isolate as *E. faecalis*. These major errors found at the first acquisition results were after solved by repeating the MALDI-TOF MS test. Phenotypically, characteristics of these bacterial genera easily could be evaluated by initial assessment such as Gram staining and colony morphology, allowing the tracking of misidentified results and after acquisition for new analysis.

Forty-four of forty-six enterococci isolates that were identified by MALDI-TOF had concordant identification with phenotypic methods. These results were similar to previous studies.[22] However, two minor errors were found for *E. gallinarum* isolates. These strains were misidentified as *E. faecium*, showing results not particularly acceptable (correctly identification at specie-level of 66.7%), although number of isolates investigated was low, similarly of other study.[29] In study of comparative genomics of enterococci strains, Palmer et al reported the divergence between *E. faecium and E. faecalis species* and identified the genetic caracteristics the motile enterococcal species *E. casseliflavus* and *E. gallinarum.* In

opposition to *E. faecalis*, wide phylogenetic divergence was observed among *E. faecium* strains.[30] This is of special interest for bacteria identification, because the MALDI-TOF is a methodology based on proteomic profile, allowing biomarkers separation by mass/charge ratio.[11] Wherefore, genomics variation among the same species distinct strains could affect the bacterial identification, since would induce changes in the microorganism protein profile. Moreover, *E. faecium* and *E. gallinarum* share similar biochemical characteristics (*Enterococcus* group II). The divergent phenotypic test between them is that *E. gallinarum* is motile and MGP (methil-alfa-D-glucopyranoside) positive, while *E. faecium* is not.[16, 31] Despite of misidentified *E. gallinarum* isolates, all *E. faecium* were correctly identificated by MALDI-TOF. This enterococci species is an emergent and challenging nosocomial pathogen, mainly vancomycin-resistant *E. faecium* (VREfm), isolated from hospitalized patients with bacteremia and endocarditis and frequently linked with multiple drugs resistance, compromising the antimicrobial therapy.[32, 33, 34, 35] Griffin et al demonstrated that the new methodology has the ability to report an accurate and rapid diagnostic test to reduce this pathogen spread in colonized patients, preventing hospital acquired infections outbreaks.[36]

One streptococci isolate was erroneously identified by MALDI-TOF (*S. gordonii* as *S. mitis/oralis*). These strains belong to Viridans and Mitis group, which comprise a significant proportion of the oropharyngeal tract normal flora.[37] Although the present study has small number of streptococci isolates (n=18), previous reports showed that Vitek MS database is more specific for *S. viridans* identification.[38] However, duo high level of the similarity between this group, analysis with a larger number of isolates should be performed to define the MALDI-TOF MS accuracy for the *S. viridans* identification.

Conclusion

MALDI-TOF MS has the potential of being an accurate tool for majority of Gram-positive cocci identification. In addition, the methodology is simple, cost-effective, fast and reliable, and can be implemented in a clinical microbiology laboratory setting, improving efficiency, turnaround time and precise bacterial identification.

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TABLES

Table 1. MALDI-TOF MS accuracy analysis for gram-positive cocci identification.

Taxon (No.)	Agreement identification between MALDI-TOF MS and conventional phenotypic method – No (%)	
	Genus level	Specie level
Staphylococcus spp. (386)	384 (99.5)	379 (98.2)
S. aureus (43)	43 (100)	43 (100)
S. epidermidis (94)	94 (100)	89 (94.7)
S. haemolyticus (106)	106 (100)	106 (100)
S. hominis (26)	26 (100)	26 (100)
S. saprophyticus (95)	94 (99)	94 (99)
Others SCoN[a] (22)	21 (95.5)	21 (95.5)
Enterococcus sp. (46)	46 (100)	44 (95.7)
E. faecalis (28)	28 (100)	28 (100)
E. faecium (8)	8 (100)	8 (100)
Others[b]	10 (100)	8 (80)
Streptococcus[c] spp. (18)	18 (100)	17 (94.4)
Micrococacceae (386)	384 (99.5)	379 (98.2)
Streptococcaceae (64)	64 (100)	61 (95.3)

[a] *S. capitis* (5), *S. caprae* (1), *S. cohnii* (3), *S. intermedius* (1), *S. lugdunensis* (1), *S. sciuri* (1), *S. warneri* (9) and *S. xylosus* (1)

[b] *E. casseliflavus* (2), *E. gallinarum* (6) and *E. hirae* (2)

[c] *S. agalactiae* (1), *S. gallolyticus* (7), *S. gordonii* (1), *S. infantarius* (2), *S. mitis/S. oralis* (3), *S. mutans* (1), *S. parasanguis* (1), *S. salivarisus* subs *salivarius* (1) and *S. sanguis* (1)

Table 2. Disagreement identifications between conventional method and MALDI-TOF MS.

Bacterial isolates (No.)	Identification provided by MALDI-TOF MS (No.)	Type of disagreement
Staphylococcus epidermidis (5)	*S. warneri* (2)	Minor Error
	S. hominis subs hominis (1)	Minor Error
	S. intermedius (1)	Minor Error
	S. saprophyticus (1)	Minor Error
Staphylococcus saprophyticus (1)	No identification	No identification
Staphylococcus warneri (1)	*Staphylococcus warneri* *Prevotella buccalis*	Unreliable identification
Enterococcus gallinarum (2)	*E. faecium* (2)	Minor Error
Streptococcus gordonii (1)	*Streptococcus mitis /Streptococcus oralis* (1)	Minor Error

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Determination of Phenotypic Profile, Antimicrobial Susceptibility and Adhesion-associated Virulence Factors by *Staphylococcus saprophyticus* Strains Recovered from Porto Alegre, RS, Brazil.

Running title: Virulence Factors in *S. saprophyticus*.

Contents Category: Pathogenicity and virulence.

Keywords: S. saprophyticus, Antimicrobial Resistance, Biofilm, Uroepithelial adherence factors.

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Abstract

Urinary tract infections (UTIs) are among the most common infections acquired in the community, representing a main source of morbidity in younger and healthy women. Uropathogens, as *Staphylococcus saprophyticus,* has been recognized as a major true Gram-positive etiologic agent of UTI, mainly by the ability to colonize the urinary tract. Therefore, the aim of the study was to evaluate the phenotypic and virulence profile of *S. saprophyticus* isolates recovered from Porto Alegre, Brazil, between 2008-2010. A total of 60.4% of *S. saprophyticus* isolates showed atypical profile by the least one phenotypic test of the reference identification. Moreover, elevated non-susceptibility (intermediate or resistance phenotypes) to trimethoprim-sulfamethoxazole and norfloxacin, and resistance to beta-lactam antibiotic were found in 10.9%, 12% and 5.1% of the strains, respectively. The adhesive property of *S. saprophyticus* to T-24 bladder carcinoma cell was 237.44 CFU/1000 cell of initial inoculum, beyond the strains to show elevated frequency of biofilm-producing (98.5%) and virulence factors associated with uroepithelial cells adhesion (*uaf*A – 100%, *aas* and *ssp* – both 98.5% and *sdr*I – 3.1%), facts not shown before in the literature. In conclusion, our findings suggested that *S. saprophyticus* strains have peculiar characteristics of virulence, allowing colonization and survival in the urinary tract environment.

Introduction

Urinary tract infections (UTIs) are common among younger and healthy women, representing a main source of morbidity and health-care costs in this age-group (Finer & Landau, 2004). Furthermore, it is one of the most common reasons to prescribe antibiotics in the world (Schollum & Walker, 2012). Although *Escherichia coli* is most frequent microorganism isolated from uncomplicated cystitis, *Staphylococcus saprophyticus* has been recognized as a major true Gram-positive uropathogens, accounting for 5.2% – 12.5% of all germs isolated from UTI (Eriksson *et al*., 2012; Filiatrault *et al*., 2012).

The adhesive properties of *S. saprophyticus* in uroepithelial cells have been studied extensively in the past (Teti *et al*., 1987; McTaggart *et al.*, 1990; Schneider & Riley, 1991). In comparation of the others commonly isolated *Staphylococcus* species, as *S. aureus*, *S. epidermidis* and *S. haemolyticus*, only *S. saprophyticus* adhere to human ureteral epithelium *in vitro*, with similar pili-like structures on their cell surfaces (Fujita *et al.,* 1992). Moreover, it has been described recently that the internalization of *S. saprophyticus* ATCC 15305 into the human urinary bladder carcinoma cell may be important for the pathogenicity of recurrent UTI caused by this microorganism, mainly due the expression of specific adhesin (Szabados *et al.,* 2008).

The proteins Adhesin-autolysin (Aas), Uro-adherence Factor A (UafA), Serine-aspartate Repeat (SdrI) and *S. saprophyticus* Surface Protein (Ssp) have been described as important potential virulence factors that enable adherence to uroepithelial cell and extracellular protein matrix of the human host, beyond persistence in the murine urinary tract model (Kuroda *et al.,* 2005; Sakinc *et al.,* 2007; Kline *et al.,* 2010). Furthermore, catheter-associated urinary tract infection, that is the most common health-care-

70 associated infection worldwide, in addition of the ability of the coagulase-negative
71 staphylococci (CNS) to establish biofilms in indwelling medical devices, a remarkable
72 charasterist of these microorganisms (Kiedrowski & Horswill, 2011; Siddiq & Darouiche,
73 2011), allows that this virulence factor also may be associated in the pathogenesis of *S.*
74 *saprophyticus* strains recovered from UTI. Therefore, the aim of the study was to evaluate
75 the phenotypic profile of bacterial identification, antimicrobial susceptibility, virulence -
76 mainly as biofilm formation and adherence to uroepithelial cells - and genotyping of putative
77 adhesion-associated factors of *S. saprophyticus* isolates recovered from Porto Alegre, Brazil.

78

79 **Material and Methods**

80

81 Bacterial Strains and Identification

82

83 The study was performed at Laboratorio de Cocos Gram Positivos da UFCSPA
84 (LCGP), Brazil. A total of 293 *Staphylococcus saprophyticus* isolates, recovered from urine
85 clinical samples between 2008 and 2010, were selected from strain collection belonging
86 LCGP. All the isolates were stored at -20 C in skin milk (Becton Dickinson S.A., USA) and
87 subcultured once on 5% blood sheep agar (bioMeriéux, Marcy I'Etoile, France) at 35°C for
88 24h before being tested.

89 One hundred eighty-seven isolates were randomly selected to perform the phenotypic
90 profile by conventional methodology proposed by Kloos & Schleifer (1975) and Bannerman
91 & Peacock (2007) for staphylococci strains. The following characteristics were tested: Gram
92 stain, catalase, susceptibility to novobiocin (5µg) (NOV), enzyme activity of urease (URE)
93 and acid production from trehalose (TRE), mannitol (MANI), mannose (MANO), xylose

(XYL), cellobiose (CEL), arabinose (ARA), maltose (MAL), lactose (LAC), sucrose (SAC), raffinose (RAF) and ribose (RIB). All tests results were obtained after incubations of 24h, 48h and 72h at 35°C.

Antimicrobial Susceptibility Testing (AST)

AST was performed by disk diffusion according CLSI (2012), using the following antimicrobials: gentamicin (10µg), ciprofloxacin (5µg), sulfametoxazole-trimethoprim (1.25/23.75 µg), cefoxitin (30µg), nitrofurantoin (300µg) and norfloxacin (10µg) (Oxoid, UK). An isolate was considered multidrug resistant (MDR) when presented resistance to three or more different antimicrobials.

In vitro Biofilm Formation Assay

Biofilm formation was determined in 131 isolates of the study by an in vitro microtiter plate assay, performed as described by Christensen *et al.* (1985), with modifications. Briefly, 180 µl of tripticase soya broth (Becton Dickinson S.A., Franklin Lakes NJ, USA) supplemented with glucose 1% was added to each well of a sterile 96-well polystyrene flat-bottom microtiter plate (TPP Techno Plastic Products, Trasadingen, Switzerland), followed by 20 µl of $1x10^8$ CFU/ml bacterial suspension. The plates were incubated for 24 h at 35 °C. After incubation, the broth was removed, all wells were washed three times with sterile saline to remove non-adherent cells and the plates were air dried in inverted position. Bacteria attached were fixed with methanol for 20 min and left to air dry overnight in an inverted position at room temperature. Adherent bacteria were stained with crystal violet 0.5% for 15 min and biofilm was eluted with ethanol for 30 min without shaking. Absorbance was

measured at 492 nm using microtiter plate reader Expert Plus – Asys Hitech GmbH (Eugendorf, Austria). The biofilm-positive strain *Staphylococcus epidermidis* ATCC 35984 was used as control of method.

Optical density (OD) results were scored and interpreted as described by Stepanovic et al. (2007). The cut-off value (ODc) was defined as three-fold the standard deviation (SD) above negative control OD and isolates were categorized as strong (OD>4ODc), moderate (2ODc<OD<4ODc), weak (ODc<OD<2ODc) producing and non-producing (OD≤ODc).

Bacterial Adherence Assay to Tissue Culture Cells

Adherence assay was performed according to procedures described previously by Kuroda *et al.* (2005) with modifications. Human T24 bladder carcinoma cells (ATCC HTB-4) were maintained in culture onto confluent epithelial cell monolayer in 24-well microtiter plates (TPP Techno Plastic Products, Trasadingen, Switzerland) by using Dulbecco's Modified Eagle's Medium (DMEM) (high glucose) supplemented with 10% v/v heat-inactivated Fetal Bovine Serum (FCS) and penicillin-streptomycin solution (Gibco-BRL, Gaithersburg, MD, USA) at 37°C in 5% carbon dioxide atmosphere. After 90% confluent growth, the tissue culture plate was washed three times with phosphate-buffered saline (PBS) (Gibco-BRL, Gaithersburg, MD, USA) to remove the DMEM containing antimicrobial solution. Bacteria cells of *S. saprophyticus* (n=17) and *E. faecalis* (n=16), randomly selected from strain collection of LCGP, were grown in Muller-Hinton broth (Becton Dickinson S.A., Franklin Lakes, USA) at 35ºC±2 overnight, followed by washing and suspending with PBS to achieve an bacterial suspension at 2 x 10^6 colony-forming units (CFU) ml $^{-1}$. Then, five-hundred microliters of bacterial suspension was added to quadruplicate for 1 h at 37ºC with gentle agitation and total number of bacteria was determined by plating (1µl) in tripticase

144 soya agar (TSA) (Becton Dickinson S.A., Franklin Lakes NJ, USA). After incubation, the
145 supernatant was removed and the wells were washed three times with 100 µl PBS and lysed
146 vigorously in 1 ml of 0.1% Triton X-100 in sterile water. To calculate the number of adherent
147 bacteria, quantitative culture (1 µl) was performed in TSA and the CFU were counted.
148 Adherence index was determined as the number of bacteria recovered after PBS washes
149 divided by the total number of bacteria of initial inoculum.
150
151 Virulence Genotyping
152
153 The *aas*, *uaf*A, *sdr*I and *ssp* nucleotide sequences were obtained from the GenBank
154 and four set of specific primers were designed to amplify this gene coding sequence (Table 1)
155 and, as positive controls, the reference strain *S. saprophyticus* ATCC 15305 (*uaf*A, *aas* and
156 *ssp*) and the clinical isolate 164 (*sdr*I) were used in the study. Genomic DNA of sixty-nine *S.*
157 *saprophyticus* strains were extracted by boiling from a bacterial suspension in TE buffer (10
158 mmol l^{-1} Tris-HCl (pH 8.0), 1 mmol l^{-1} EDTA), as described by Yang *et al.* (2008) with
159 modification. After this step, an aliquot of 1 µl of this suspension was added to 24 µl of PCR
160 mixture containing Tris-HCl buffer (pH 8.4), 1.5 mM MgCl2, 0.2 mM of each
161 deoxynucleoside triphosphate (dATP, dUTP, dGTP, and dCTP), 1.25 U of Platinum Taq DNA
162 polymerase (Invitrogen, Carlsbad CA, USA) and 0.5 µM of each primer (IDT Integrated DNA
163 Technologies, Inc, USA).
164 The amplification was performed in a LifePro Thermal Cycler (Hangzhou Bioer
165 Technology Co. Ltda, Hangzhou, China) beginning with an initial denaturation step at 94°C
166 for 5 min followed by 30 cycles of 94°C 1 min, 52°C 1 min, 72°C 1 min, and final extension
167 at 72°C for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis (Gibco-
168 BRL, Gaithersburg, MD, USA) stained with ethidium bromide and visualized under UV light.

169

170 Statistical Analysis

171

172 In this study, descriptive measures were calculated for all the variables. From the
173 phenotypic bacterial identification, atypical strains were defined as those that showed
174 different results for tests that are considered not variable by the reference method (Bannerman
175 & Peacock, 2007; Iorio *et al.* 2007). Susceptibility results were analyzed as frequencies, and
176 antibiotic resistance was compared at the time of sample isolation. Moreover, chi-square test
177 or Fisher's exact test was used to evaluate an association among bacterial isolates with
178 decreased susceptibility (defined as strains that showed at least intermediate or resistant
179 phenotypes by disk-diffusion methods) and the biofilm formation.

180 Statistical difference of the bacterial adherence between *S. saprophyticus* and *E.*
181 *faecalis* strains to T24 bladder cell was calculated by non-parametric Mann-Whitney U-test.
182 All analyses were performed using SPSS software (ver. 16.0; Chicago, IL, USA) and the
183 threshold for statistical significance was $P < 0.05$.

184

185 **Results**

186

187 A total of 113 (60.4%) of 187 *S. saprophyticus* isolates showed atypical profile by the
188 least one phenotypic test of the reference identification. However, the frequency of positive
189 reaction in relation to each one of the 13 characteristic evaluated is in agreement with
190 literature, except to ribose acid production. The summary of phenotypic profile is shown in
191 Table 2.

192 Based on susceptibility results, gentamicin (99.6%), nitrofurantoin (98.6%) and
193 ciprofloxacin (95.4%) were the most effectivity antibiotics in this study. Norfloxacin and

194 sulfametoxazole-trimethoprim showed reduced susceptibilities compared with others
195 antimicrobial agents (Fig. 1a). Moreover, the *S. saprophyticus* showed resistance to beta-
196 lactam antibiotic (by screening with cefoxitin disks) in 5.1% of clinical isolates. MDR was
197 not found among the strains; however, 68 (23.3%) of 292 isolates showed reduced
198 susceptibility (resistant or intermediary phenotypes at least one antimicrobial agent tested). At
199 the 3 years of isolates collection, antimicrobial resistance to ciprofloxacin and norfloxacin
200 decreased (Fig. 1 b), on the other hand strains resistant to sulfametoxazole-trimethoprim and
201 nitrofurantoin remained steady. One hundred twenty-nine (98.5%) isolates were biofilm-
202 positive in the microtiter plate assay (Fig. 2a). Most of the isolates were grouped as strong and
203 moderate biofilm-producers (78.6%) and there was no association among biofilm phenotypes
204 and isolates with reduced susceptibility to antibiotics tested in the study (p=0.871). The
205 adhesive properties of *S. saprophyticus* strains was lower than *E. faecalis* in T-24 bladder
206 carcinoma cell line, however, there was no statistical difference in Adherence Index between
207 the species (237.4±273.6 and 394.7±398.4 CFU/1000 cell of initial inoculum) respectively,
208 (p=0.360) (Fig. 2b), with the percentage of adherence ranging from 13.3 to 815.6 CFU/1000
209 cell of initial inoculum.

210 The numbers of *S. saprophyticus* carrying genes associated with adherence-virulence
211 factors was performed to 69 clinical isolates. All the strains carry surface protein UafA
212 (100%), and was found elevated prevalence of Aas and surface-associated lipase Ssp (both
213 98.5%). However, collagen binding SdrI was detected in 3.1% by PCR.

214

215 **Discussion**

216

217 *Staphylococcus saprophyticus* have been the most frequent causative bacterial specie

218 of uncomplicated UTI among the Gram-positive cocci and the majority of infections occur in
219 young, healthy and sexually active women (Raz *et al.,* 2005). Historically, the *S.*
220 *saprophyticus* isolated from the urine samples has been recognized as etiologic agents of UTI,
221 not as contaminant (Hovelius & Mårdh, 1984); however, it is known that a heterogeneous
222 group of CNS composed of several different species show resistance to novobiocin
223 (McTaggart & Elliott, 1989), the major characteristic of *S. saprophyticus* identification, due
224 intrinsic resistance by mutation points in the GyrB subunit of DNA gyrase (Vickers *et al.,*
225 2007).

226 Actually, *Staphylococcus* sp species as *S. cohnii, S. sciuri* and *S. xylosus* has been
227 recognized of emerging pathogens, with outbreak caused by linezolid-resistant strains in
228 intensive care unit (ICU) (Petinaki *et al.,* 2009), wound infection by multidrug-resistant
229 strains (Coimbra *et al.,* 2011) and brain abscess (Akhaddar *et al.,* 2010), respectively.
230 Moreover, these bacterial species also were described in UTI or colonizing the human
231 urogenital tract (Orrett & Shurland, 1998; Stepanovic *et al.,* 2005). Common characteristic
232 among these species is the novobiocin resistance (Iorio *et al.,* 2007), which may be
233 erroneously identified by automated methods as *S. saprophyticus*, such previously reported in
234 literature (Kim *et al.,* 2008).

235 In the study, the phenotypic profile of *S. saprophyticus* was evaluated by conventional
236 identification method, showing high prevalence of atypical strains. Our results show elevated
237 variability among the strains, that may result in misidentified isolates recovered from the
238 urine samples, mainly to *S. cohnii* subs *urealyticum* (both specie are coagulase-negative,
239 novobiocin resistant and urease-positive) (Bannerman & Peacock, 2007). Despite of the
240 60.4% of clinical isolates have showed atypical biochemical profile, the positivity frequencies
241 of the phenotypic tests is in agreement with the literature, in exception of acid production by
242 ribose, presenting as variable test (see Table 2). Moreover, the phenotypic characterization of

S. saprophyticus strains enable numerical approaches to identify microorganisms, since the frequency of positivity tests may generate a biochemical database, allowing to calculate the probability an unknown isolate belong to *S. saprophyticus* specie by Bayes' theorem approach (Flores et al 2009). Therefore, the biochemical profile obtained from *S. saprophyticus* isolates, to our knowledge, is important for staphylococcal identification of strains recovered from urine samples.

The routine AST to *S. saprophyticus* isolates recovered from urine samples is not advised by the CLSI (2012) guidelines, since the antibiotics as nitrofurantoin, trimethoprim-sulfamethoxazole and fluorquinolones – ciprofloxacin and norfloxacin - achieve satisfactory concentrations in urine to treat UTI. Furthermore, *Escherichia coli* is the most frequent microorganism isolated from UTI, and the antibiotic resistance rates are higher than others uropathogens (Abu-Taha & Sweileh, 2011; Filiatrault *et al.,* 2012; Schmiemann *et al.,* 2012). In the study, *S. saprophyticus* isolates showed high susceptibility to antibiotics commonly used to treat UTIs (88.0% to 98.6%), similar to a previous data, where resistance rate was lower mainly to fluorquinolones and nitrofurantoin (Marzouk *et al.,* 2009). There was elevated non-susceptibility rate to trimethoprim-sulfamethoxazole and norfloxacin antimicrobial agents (10.9% and 12% showed intermediate or resistance phenotypes, respectively). In an ARESC (Antimicrobial Resistance Epidemiological Survey on Cystitis) study performed by Schito *et al.* (2009), resistant strains were rare in *S. saprophyticus*, with the exception of trimethoprim-sulfamethoxazole (10.2%). The key question about the administration of these antimicrobial drugs is the empirical use to treat UTI. Recent studies have demonstrated that trimethoprim-sulfamethoxazole and fluorquinolones follow a worldwide tendency of increasing resistance and it should be avoided as first-line empirical treatment of UTI (Araújo *et al.,* 2011; Neuzillet *et al.,* 2012). In view of these data, susceptibility profile of *S. saprophyticus* isolates can therefore influence the treatment of UTI,

268 unresponsive appropriately to empiric antimicrobial therapy.

269 Isolates of *S. saprophyticus* resistant to beta-lactam antimicrobial agents by acquisition
270 of *mec*A gene has been reported in literature, with great genetic diversity among the types of
271 staphylococcal cassette chromosome *mec* (SCCmec) (Söderquist & Berglund, 2009; Zong *et*
272 *al.,* 2011). In 3 years of bacterial isolation, 5.1% of strain showed beta-lactam resistance by
273 screening of cefoxitin disks, that have been demonstrated high sensitivity and specificity to
274 detect methicillin-resistant *S. saprophyticus* (MRSS) (John *et al.,* 2009). Such common
275 inhabitant of the urinary and gastrointestinal tract, this uropathogen can be a reservatory to
276 horizontal resistance gene transfer among bacterial species, a characteristic of CNS (Hanssen
277 *et al.,* 2004; Malachowa & DeLeo, 2010). Therefore, special attention in the diagnostic of
278 these resistant strains should be performed for epidemiological purposes, monitoring the
279 MRSS isolation rate recovered from UTI.

280 The ability to produce biofilm is a fundamental factor in the pathogenesis of infections
281 by CNS, mainly to medical device-related infections. Biofilms are surface-attached
282 agglomerations of bacteria that are embedded in an extracellular matrix (exopolysaccharide
283 polysaccharide intercellular adhesin – PIA), that is synthesized by the gene products of the *ica*
284 (intercellular adhesion) operon, and provide protection from antimicrobial agents and
285 mechanisms of host defense (Otto, 2012), resulting in infections that are extremely difficult
286 to treat, frequently chronic and recurrent, making them a clinical problem (Brooks &
287 Jefferson, 2012).

288 Previous studies reported that *S. saprophyticus,* by Congo red agar (CRA) screening
289 methodology, was able to form biofilm despite the low frequency these strains to show this
290 virulence factor (Hjelm & Lundell-Etherden, 1991; Riley & Schneider, 1992). In the present
291 study, high prevalence of biofilm-formers isolates was found (98.5%), with 78.6% showing
292 strong and moderate phenotypes (see Fig. 2a). As the microtiter plates assay is more accurate

293 and reproducible technique than CRA, that only detect the extracellular adhesin
294 polysaccharide (Mathur *et al.,* 2006; Tamai *et al.,* 2011), this difference between the results is
295 likely due the employed methods for determining the biofilm, which may underestimate the
296 prevalence of biofilm in these clinical isolates, since the *S. saprophyticus* strains do not have
297 the *ica* operon in their genome and do not produce PIA (Kuroda *et al.,* 2005; Park *et al.,*
298 2010). Therefore, our results reveal the high tendency of *S. saprophyticus* to form biofilm,
299 meanwhile, the role of this virulence factor has not yet been established, and further studies
300 are needed to define and better the understanding about biofilm in the pathogenesis of this
301 uropathogen in UTI.

302 In addition to *S. saprophyticus* isolates show the ability to adhere to the abiotic
303 surface, as demonstrated by microtiter plate assay, the results of the present study indicated
304 that these strains also adhere to the uroepithelial cells (see Fig. 2b). In comparison of *E.*
305 *faecalis*, other frequent Gram-positive cocci isolated from UTI (Magliano *et al.,* 2012;
306 Schmiemann *et al.,* 2012), there was no statistically significant differences between adhesive
307 properties of the species. A singular characteristic of *S. saprophyticus* strains facing others
308 commonly isolated staphylococci species, as *S. aureus* and *S. epidermidis,* is the presence of a
309 abundant transporter systems to facilitate adaptation to human urine, with additional sets of
310 osmoprotectant transport systems that may contribute to the prompt balancing of intracellular
311 osmotic pressure against highly variable osmotic change in urine (Kuroda *et al.,* 2005). This
312 physiologic property of *S. saprophyticus*, beyond specific adhesins, likely enable that clinical
313 isolates have the ability to adhere and grow persistently in the urinary tract.

314 Staphylococci strains harbor a variety of adhesins in their cell surfaces that mediate
315 attachment to a several host factors as extracellular matrix proteins like collagen, fibrinogen
316 and fibronectin, allowing the adherence and colonization of human host cells (Heilmann,
317 2011; Krishnan & Narayana, 2011). In *S. saprophyticus*, cell wall-associated protein Aas,

UafA and SdrI contain domains with fibronectin-collagen binding and haemagglutinating activities that contribute to adhesion to the urinary tract, which would be a severe environment for bacterial colonization due to intensive urine flux (Hell *et al.,* 1998; Matsuoka *et al.,* 2011; Sakinc *et al.,* 2009). Other molecule, Ssp is the major surface-associated protein found in this staphylococci specie, in which produce a fuzzy fibrillar surface layer in bacterial cells. Previous study suggest that Ssp does not function as an adhesin, but shows lipase activity that may contribute to persistence of the microorganism by providing a source of energy or by facilitating adherence in host cell (Sakinc *et al.,* 2005). In the study, clinical isolates show high prevalence of these potential adhesion-associated factors in their genome, with the exception of *sdr*I (3.1%). These finding are according with a previous report, which evaluated *S. saprophyticus* isolates from patients in Germany (Kleine *et al.,* 2010). The knowledge about the frequency of these virulence factors in clinical isolates allows to evaluate the mechanisms used by this uropathogen in UTI. Further studies are needed to measure the contribution of these adherence proteins in *S. saprophyticus* pathogenesis, with more attention to host and urinary environment factors that allow the uroepithelial tissue colonization.

In conclusion, *S. saprophyticus* recovered from Porto Alegre, Brazil, showed high phenotypic variability and a considerable number of strains with decreased antimicrobial susceptibility. Moreover, elevated frequency of biofilm-producing and virulence factors associated with uroepithelial cells adhesion were found in clinical isolates, facts not shown before in the literature. Our findings suggested that *S. saprophyticus* strains have peculiar characteristics of virulence, allowing colonize and survive in the urinary tract environment.

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TABLES

Table 1: Sequences of Oligonucleotide Primers to Detection of Virulence Factors in *Staphylococcus saprophyticus*.

Gene	Genbank accession number	Primer	Sequence	Amplicon size (bp[£])
aas	AP008934.1[*]	aas−F	CAAATCAAAACACACCAATCG	130
		aas−R	CAAGGGACAGCATAAAGACCA	
*uaf*A	AP008934.1[#]	uafA−F	TTTTCGGGGATTGGTGTATT	350
		uafA−R	GTGCTGTTCGCTTGTGTAGG	
*sdr*I	AF402316.1	sdrI−F	ACAAAATGATGACGAAAAAGGT	177
		sdrI−R	GATGGCGACGGAGTGTAGTT	
ssp	AY551101.1	ssp−F	CAACAAACCGCCTAACTGAT	235
		ssp−R	GCTTTCTCTGCTGTGGGATT	

[£] Base pairs.
[*] Genome region: 151261 to 158211 bp.
[#] Genome region: 1809572 to 1813963 bp.

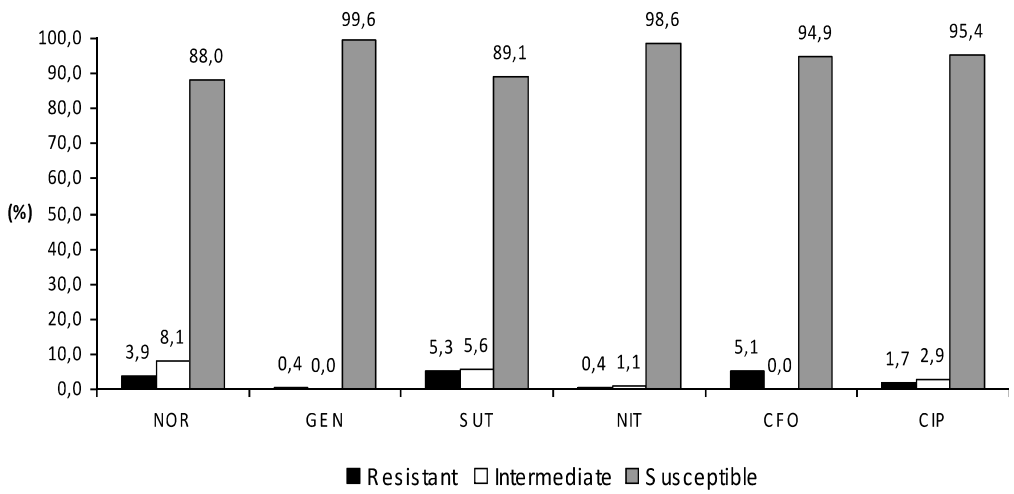
Table 2: Phenotypic profile of *Staphylococcus saprophyticus* clinical isolates of the study.

Clinical isolates with:	Phenotypic test *												
	NOV	URE	TRE	MANI	MANO	XYL	CEL	ARA	MAL	LAC	SAC	RAF	RIB
Positive reaction (n)	184	187	175	121	19	3	3	3	169	64	173	1	84
Negative reaction (n)	2	0	12	66	168	184	184	184	18	123	14	186	103
Total (n)	187	187	187	187	187	187	187	187	187	187	187	187	187
Frequencie (%)	98,4	100,0	93,6	64,7	10,2	1,6	1,6	1,6	90,4	34,2	92,5	0,5	44,9
S. saprophyticus #	R	+	+	v	-	-	-	-	+	v	+	-	-

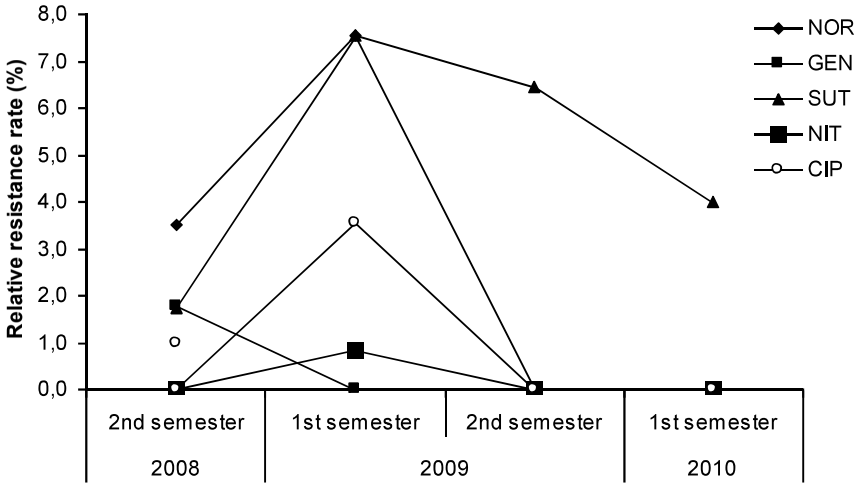
* R – novobiocin resistance; (+) ≥90% of strains with positive reaction; (-) ≤10% of strains with negative reaction; (v) positive reaction are achieved with 11 to 89% of strains; (NOV) susceptibility to novobiocin; (URE) enzyme activity of urease; (TRE) trehalose; (MANI) mannitol; (MANO) mannose; (XYL) xylose, (CEL) cellobiose; (ARA) arabinose; (MAL) maltose; (LAC) lactose; (SAC) sucrose; (RAF) raffinose and (RIB) ribose.

Kloos & Schleifer (1975); Bannerman & Peacock (2007).

FIGURES

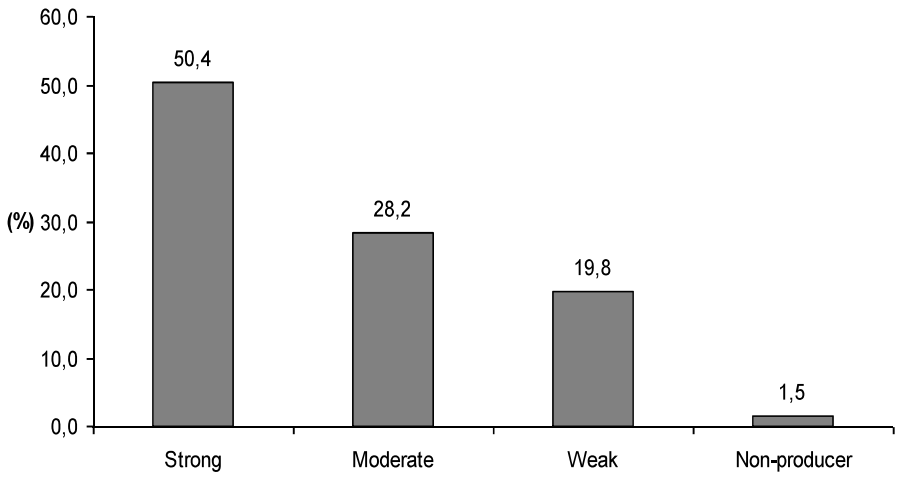


(a)

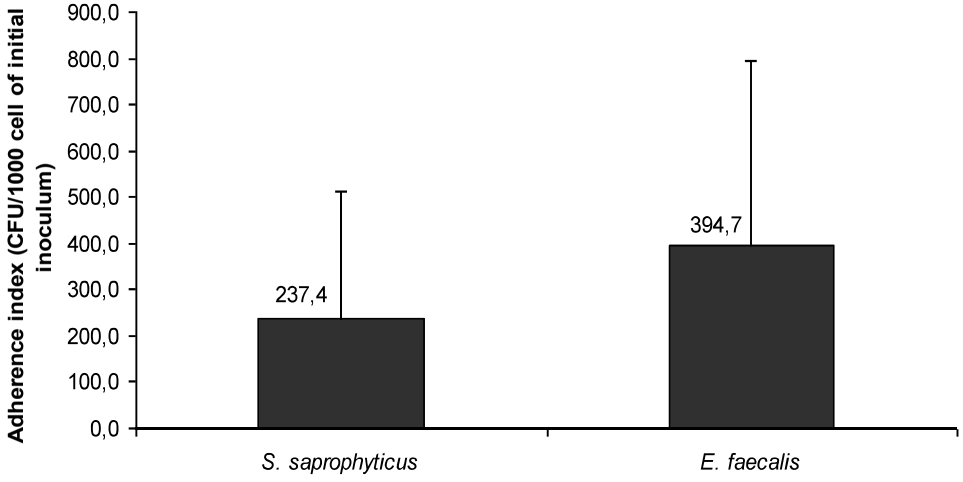


(b)

Figure 1: **(a)** Antimicrobial susceptibility profile of 292 *S. saprophyticus* strains isolated in Porto Alegre, Brazil. **(b)** Relative resistance rate (number of resistant microorganisms divided by the total of strains isolated from the period) of the antimicrobial agents tested in the study.



(a)



(b)

Figure 2: **(a)** Biofilm formation by *S. saprophyticus* isolates (n=131). **(b)** Comparative adhesive property of *S. saprophyticus* and *E. faecalis* strains in T-24 bladder cell line.

CONCLUSÕES

A partir dos resultados obtidos, conclui-se:

O sistema automatizado Vitek 2 apresentou elevada acurácia na identificação dos principais gêneros de cocos Gram-positivos do estudo bem como para as espécies bacterianas comumente isoladas no laboratório de microbiologia clínica, como *S. aureus* e *E. faecalis*. Entretanto, em nível de espécie, o desempenho do sistema Vitek 2 na identificação de micro-organismos menos frequentemente isolados foi aquém do esperado (ver manuscrito I). Para isolados de *Staphylococcus* do grupo *saprophyticus* (n=15), a acurácia obtida foi de 86,7%, com dois isolados de *S. saprophyticus* identificados como *S. warneri* e *S. cohnii* subs *urealyticus*.

A metodologia MALDI-TOF apresentou elevada acurácia na identificação de cocos Gram-positivos pertencentes ao banco de amostras do Laboratório de Cocos Gram-positivos da UFCSPA. Concordância em nível de espécie foi obtida em 98,2% dos isolados de *Staphylococcus*, com 99% das cepas de *S. saprophyticus* identificadas corretamente, além de todas as amostras de outras espécies de *Staphylococcus* do grupo *saprophyticus*. Quando comparada à metodologia fenotípica referência, elevado número de isolados de *S. saprophyticus* apresentaram perfil atípico de identificação.

Esses dados sugerem que isolados de *S. saprophyticus* apresentam expressão metabólica variável, sendo um fator limitante na identificação fenotípica de suas cepas, diferentemente da aproximação proteômica proporcionada pela metodologia MALDI-TOF, na qual evidenciou perfis de espectro de massa típicos entre as espécies estudadas. Esse método apresentou elevada especificidade na diferenciação entre espécies pertencentes ao grupo *saprophyticus*.

Apesar de não ser recomendado a testagem de amostras de *S. saprophyticus* para

avaliação do perfil de suscetibilidade aos antimicrobianos (documento do CLSI), o presente estudo proporcionou o conhecimento epidemiológico de como esses micro-organismos isolados na cidade de Porto Alegre respondem aos principais antibióticos utilizados no tratamento de ITU. Especial atenção deve ser direcionada no uso de antimicrobianos como norfloxacina e trimetroprim-sulfametoxazol, os quais apresentaram reduzido nível de suscetibilidade nas amostras testadas.

A formação de biofilme como um fator de virulência de isolados de *S. saprophyticus* tem-se mostrado negligenciada na literatura científica. No estudo, esse fator de virulência se apresentou como uma característica comum entre amostras clínicas, no qual é um potencial fator para o processo de aderência ao uro-epitélio. A contribuição da formação de biofilme na patogênese das infecções por *S. saprophyticus* deve ser melhor elucidada em estudos posteriores.

Os isolados de *S. saprophyticus* apresentaram aderência à linhagem uro-epitelial T-24, semelhantemente aos isolados de *E. faecalis*, outro agente comum recuperado de ITU não-complicadas. Além disso, a elevada prevalência de determinantes genéticos associados à adesão ao tecido do hospedeiro foi encontrada em *S. saprophyticus*. Os resultados obtidos proporcionam um entendimento de quão aptos esses isolados estão para aderir e colonizar o ambiente urinário.

ANEXO I – Co-autoria em trabalhos publicados.

Reiter KC, Sambrano GE, Villa B, Paim TG, de Oliveira CF, d'Azevedo PA. Rifampicin fails to eradicate mature biofilm formed by methicillin-resistant Staphylococcus aureus. Rev Soc Bras Med Trop. 2012 Jul-Aug;45(4):471-4.

Source

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Abstract

INTRODUCTION:

Antimicrobial activity on biofilms depends on their molecular size, positive charges, permeability coefficient, and bactericidal activity. Vancomycin is the primary choice for methicillin-resistant Staphylococcus aureus (MRSA) infection treatment; rifampicin has interesting antibiofilm properties, but its effectivity remains poorly defined.

METHODS:

Rifampicin activity alone and in combination with vancomycin against biofilm-forming MRSA was investigated, using a twofold serial broth microtiter method, biofilm challenge, and bacterial count recovery.

RESULTS:

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration for vancomycin and rifampicin ranged from 0.5 to 1mg/l and 0.008 to 4mg/l, and from 1 to 4mg/l and 0.06 to 32mg/l, respectively. Mature biofilms were submitted to rifampicin and vancomycin exposure, and minimum biofilm eradication concentration ranged from 64 to 32,000 folds and from 32 to 512 folds higher than those for planktonic cells, respectively. Vancomycin (15mg/l) in combination with rifampicin at 6 dilutions higher each isolate MIC did not reach in vitro biofilm eradication but showed biofilm inhibitory capacity (1.43 and 0.56log10 CFU/ml reduction for weak and strong biofilm producers, respectively; $p<0.05$).

CONCLUSIONS:

In our setting, rifampicin alone failed to effectively kill biofilm-forming MRSA, demonstrating stronger inability to eradicate mature biofilm compared with vancomycin.

Reiter KC, Villa B, da Silva Paim TG, Sambrano GE, de Oliveira CF, d'Azevedo PA. Enhancement of antistaphylococcal activities of six antimicrobials against sasG-negative methicillin-susceptible Staphylococcus aureus: an in vitro biofilm model. Diagn Microbiol Infect Dis. 2012 Oct;74(2):101-5.

Source

Health Sciences Post-Graduate Program, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil. kelicrisr@gmail.com

Abstract

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

Reiter KC, DA Silva Paim TG, DE Oliveira CF, D'Azevedo PA. High biofilm production by invasive multiresistant staphylococci. APMIS. 2011 Nov;119(11):776-81.

Source

Health Sciences Post-graduate Program, Universidade Federal de Ciências da Saúde de Porto Alegre, Brazil. kelicrisr@gmail.com

Abstract

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

Reiter K, Villa B, Paim TG, Oliveira CF, d'Azevedo PA. Inhibition of biofilm maturation by linezolid in methicillin-resistant Staphylococcus epidermidis clinical isolates: comparison with other drugs. J Med Microbiol. 2012 Nov 15. [Epub ahead of print]

Source

1 Universidade Federal de Ciencias da Saude de Porto Alegre;

Abstract

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

ANEXO II – Resumos e pôsteres apresentados em eventos científicos

Tema Livre

PAIM, TGS; CANTARELLI, W; AZEVEDO, PAD. IMPORTÂNCIA DE UM SISTEMA AUTOMATIZADO ATUALIZADO NA ROTINA DE LABORATÓRIO DE MICROBIOLOGIA CLÍNICA. J Bras Patol Med Lab. Volume 48, Número 4, agosto de 2012. Suplemento 46º Congresso Brasileiro de Patologia Clínica/Medicina Laboratorial.

REITER, KC; **PAIM, TGS**; OLIVEIRA, CF; AZEVEDO, PAD. ESPECTROMETRIA DE MASSAS NA IDENTIFICAÇÃO DE STAPHYLOCOCCUS SPP. NO LABORATÓRIO DE MICROBIOLOGIA CLÍNICA. J Bras Patol Med Lab. Volume 48, Número 4, agosto de 2012. Suplemento 46º Congresso Brasileiro de Patologia Clínica/Medicina Laboratorial.

Resumo

K. C. Reiter, B. Villa, **T. G. S. Paim**, C. F. Oliveira, P. A. d'Azevedo. Linezolid Inhibition of Mature Biofilm In Methicillin-resistant Staphylococcus epidermidis Clinical Isolates: Comparation Wiyh Other Drugs. 112th General Meeting. American Society for Microbiology (ASM), June 2012, San Francisco, California.

Apresentação de Pôster

MARKUS-LOPES, PG; REITER, KC; **PAIM, TGS**;OLIVEIRA, CF; D'AZEVEDO, PA. IDENTIFICAÇÃO DE ESPÉCIES DO GÊNERO ENTEROCOCCUS E DO COMPLEXO STREPTOCOCCUS BOVIS/EQUINUS UTILIZANDO A METODOLOGIA DE MALDI-TOF. XXI Congresso Latino-Americano de Microbiologia. Santos, SP - Brasil. 2012.

ANEXO III – Parecer Consubstanciado do CEP-UFCSPA

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Caracterização fenotípica e análise de fatores de virulência em staphylococcus saprophyticus.

Pesquisador Responsável Pedro D'Azevedo **Parecer** 1729/12

Data da Versão 09/12/2011 | **Cadastro** 877/11 | **Data do Parecer** 21/06/2012

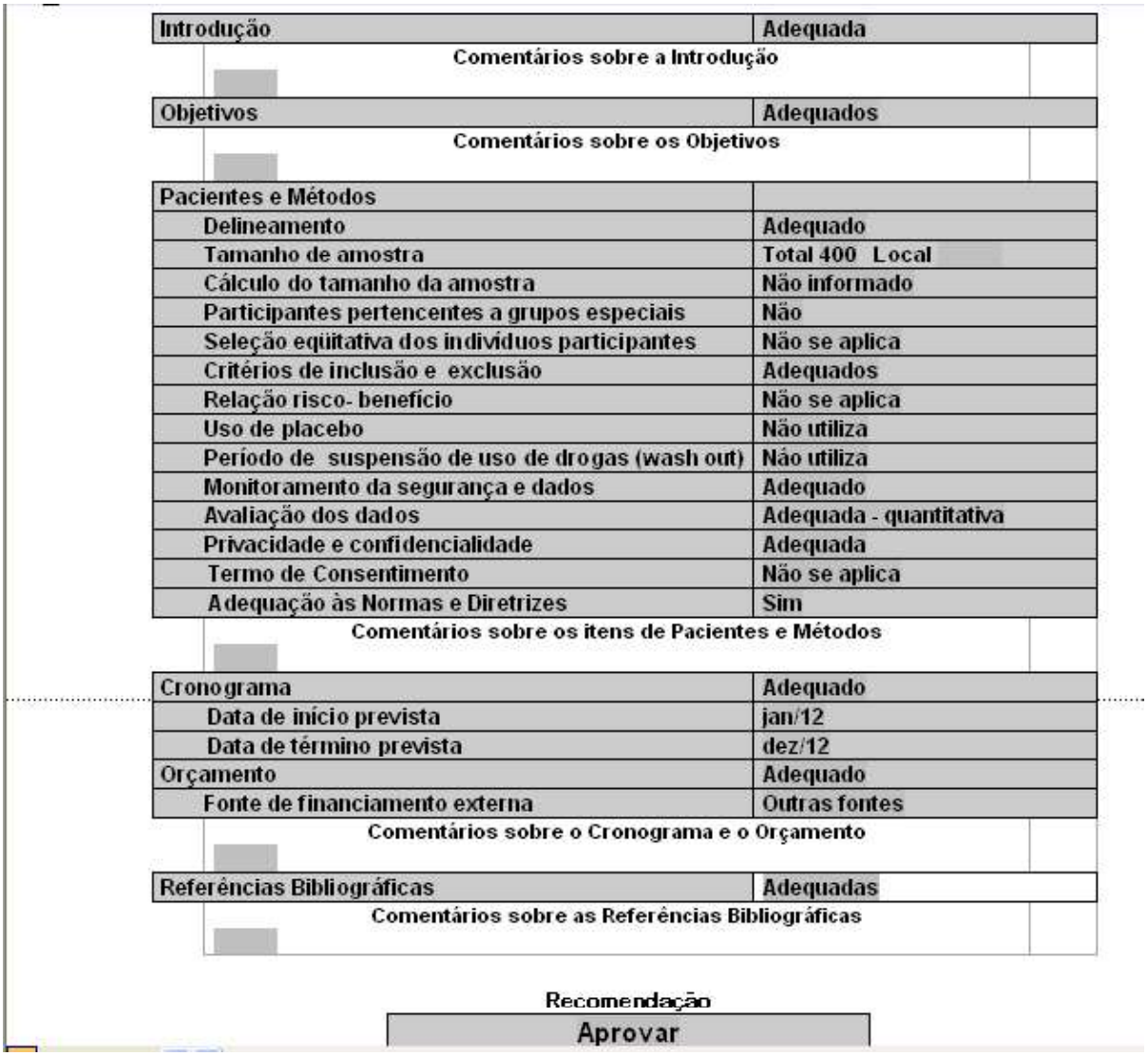
Grupo e Área Temática Classificação utilizada pela CONEP

Objetivos do Projeto
Caracterizar fenotipicamente isolados de S.s., avaliar o perfil de suscetibilidade aos antimicrobianos oriundos do sitio urinário e pesquisar os principais fatores de virulência dos isolados.

Sumário do Projeto
Isolados de S.s oriundos de uroculturas serão submetidos a testes bioquimicos de identificação, perfil de suscetibilidade aos antimicrobianos, determinação da concentração inibitória mínima (CIM) à oxacilina, ensaio de formação de biofilme, ensaio de aderência em cultura celular e RT-qPCR para determinar a contribuição de genes de fatores de virulência na capacidade adesiva dos isolados em linhagem uroepitelial.

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	Não
Condições para realização	Adequadas

Comentários sobre os itens de Identificação



Introdução	Adequada
Comentários sobre a Introdução	
Objetivos	Adequados
Comentários sobre os Objetivos	
Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total 400 Local
Cálculo do tamanho da amostra	Não informado
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	Adequado
Avaliação dos dados	Adequada - quantitativa
Privacidade e confidencialidade	Adequada
Termo de Consentimento	Não se aplica
Adequação às Normas e Diretrizes	Sim
Comentários sobre os itens de Pacientes e Métodos	
Cronograma	Adequado
Data de início prevista	jan/12
Data de término prevista	dez/12
Orçamento	Adequado
Fonte de financiamento externa	Outras fontes
Comentários sobre o Cronograma e o Orçamento	
Referências Bibliográficas	Adequadas
Comentários sobre as Referências Bibliográficas	

Recomendação

Aprovar

ANEXO IV – Instruções para submissão de artigos científicos para publicação em periódicos

Instrução aos autores para submissão de artigo científico no periódico *Diagnostic Microbiology and Infectious Disease*

Diagnostic Microbiology and Infectious Disease

Instructions to Authors

As of 01 June 2005, all new manuscripts must be submitted through the*Diagnostic Microbiology and Infectious Disease* online submission and reviewWeb site (http://ees.elsevier.com/dmid/). Authors are requested to submitthe text, tables, and artwork in electronic form (not as a PDF) to this address. In an accompanyingletter, authors should state that the manuscript, or parts of it, havenot been and will not be submitted elsewhere for publication. Authors arehighly encouraged to include a list of three or more potential reviewers fortheir manuscript, with complete contact information.

Submission items include a cover letter (save as a separate file for upload), suggested reviewers, themanuscript (including title page, abstract, manuscript text, references, andtable/figure legends), tables, and figures. Revised manuscripts should also beaccompanied by a unique file (separate from the covering letter) with responsesto reviewers' comments. The preferred order of files is as follows: cover letter,suggested reviewers, response to reviews (revised manuscripts only), manuscript file(s), table(s),figure(s). Files should be labeled with appropriate and descriptive file names(e.g., SmithText.doc, Fig1.eps, Tables3.doc). Upload text, tables and graphicsas separate files. (You can compress multiple figure files into a Zip file and upload that in one step; the system will then unpack the files and prompt you to name each figure.) Do not import figures or tables into the text document

and do not upload your text as a PDF.Complete instructions for electronic artwork submission can be found on theAuthor Gateway, accessible through the journal home page. Your figures will be tested by an artwork quality check tool; you will be asked to view the results before you can complete your submission. Your figures can move into review if not up to production standards, but you should be prepared to provide better quality figures should we express interest in your manuscript.

Authors who are unable to provide an electronic version or have othercircumstances that prevent online submission must contact the Editorsprior to submission to discuss alternate options. The Publisher andEditors regret that they are not able to consider submissions that do notfollow these procedures.

Please note, although the Elsevier Editorial System Registration page asks if you are available for reviews, we are not currently seeking new reviewers as members of the Editorial Board. All other correspondence should be addressed to the Editor in Chief:Ronald N. Jones, M.D., Editorial Office, *Diagnostic Microbiology and InfectiousDisease*, Suite A, 345 Beaver Kreek Centre, North Liberty, Iowa 52317, USA.

Manuscripts

Papers may be submitted that are full-length articles (including subject review articles), orshort notes. Manuscripts are accepted with the understanding that they are original, unpublishedwork and are not being submitted elsewhere. All manuscripts are subjected to peer review by theeditors, by members of the Editorial Board, or by other qualified reviewers.

Papers must be accompanied by a letter signed by the corresponding author indicating thatthey have read and are familiar with the current "Instructions to Authors" (published ineach issue) and will comply with the instructions and stated conditions. The letter should indicatethat all of the named authors and acknowledged parties have agreed to the submitted

draft of thepaper or, as providers of personal communications, have consented to their inclusion, and that thecontent/submission is original/unpublished and has not been simultaneously submitted to anothermedical journal. Furthermore, previous or other publication of any or part of the content of themanuscript (including conference or congress proceedings, letters, and brief communications)must be declared on the title page. Failure to comply with the above mentioned policies mayresult in a 3-year suspension of publishing privileges in this journal.

Manuscripts should be submitted via the Elsevier Editorial System, in English, double-spaced, and, where appropriate, divided into *Introduction; Materials and Methods,*which should include sufficient technical information so that experiments can be repeated andwhich should give sources of unusual chemicals, reagents, equipment, or microbial strains;*Results,* which should describe the design of the experiments as well as the resultsusing text, tables, or figures; and *Discussion,* which should provide an interpretation ofthe results in relation to previously published work. An *abstract* is required for allpapers; it should be 150 words or less for full-length papers and 50 words or less for notes. Papers for the Notes category, which is intended for the presentation of brief observations(including instructive case reports), that do not warrant full-length papers, should not contain anysection heading and should not exceed 1,000 words.

The first page of the manuscript should include: title, running title of not more than 45characters and spaces, full names of all authors, address of the institution at which the work was performed, and the corresponding author's full address, telephone number, and FAX number. Any change of address by any of the authors should also be noted. Any footnotes to the textshould be numbered using Arabic numerals and should be typed on the page of the manuscript onwhich they are referred to.

Tables

Tables should be on separate files and numbered using Arabic numerals. Each tableshould have a brief title with detailed information appearing as footnotes bearing superscript,lower-case letters. Vertical rules should be avoided.

References

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications should not be in the reference list, but may be mentioned in the text. Citation of a reference as 'in press' implies that the item has been accepted for publication. *Diagnostic Microbiology and Infectious Disease* uses the standard 'Vancouver' system with name and year in the text.

Text: All citations in the text should refer to:

1. *Single author*: the author's name (without initials, unless there is ambiguity) and the year of publication;

2. *Two authors*: both authors' names and the year of publication;

3. *Three or more authors*: first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: "as demonstrated in wheat (Allan, 1996a, 1996b, 1999; Allan and Jones, 1995). Kramer et al. (2000) have recently shown ...."

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication.

Examples:

Reference to a journal publication: Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2000;163:51-9.

Reference to a book: Strunk Jr W, White EB. The elements of style. 3rd ed. New York: Macmillan, 1979.

Reference to a chapter in an edited book: Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age. New York: E-Publishing Inc.; 1994. p. 281-304.

Note shortened form for last page number. e.g., 51-9, and that for more than 6 authors the first 6 should be listed followed by 'et al.' For further details you are referred to "Uniform Requirements for Manuscripts submitted to Biomedical Journals" (J Am Med Assoc 1997;277:927-34), see alsohttp://www.nlm.nih.gov/tsd/serials/terms_cond.html

Figures

All manuscripts must be accompanied by complete artwork. Each figure must be on a separate file. Electron micrographs should beof sufficient contrast to withstand reduction and printing at the journal page size. The cost ofprinting color photographs must be borne by the author. Any graphs, charts, or diagrams shouldbe finished drawings, using type size large enough to be read easily when reduced to page size.

Proofs and Reprints

The corresponding author will receive page proofs, which should be corrected and returned within 48 hours of receipt. Corrections are limited to printer's errorsand no substantial author's changes will be made. Reprints may be ordered at the price listed onthe order form accompanying the proofs.

Style and Nomenclature

Authors should use the *CBE* (Council of Biology Editors) *StyleManual,* 5th ed., as a general guide for style. The names of chemical compounds shouldconform with *Chemical Abstracts* and its indexes or *The Merck Index,*11th ed. Enzymes and biochemical terms should bear the recommended, trivial name listed in thelatest edition of*Enzyme Nomenclature.* Whenever possible, use generic drug names. Binary names consisting of a generic name and a species name must be used for allmicroorganisms. Names of general and higher categories may be used alone. However, a speciesname must be preceded by the complete generic name the first time that it is used in themanuscript. Following this, the generic name should be abbreviated to the initial capital letter (forexample, *S. aureus*), provided there can be no confusion with other genera oforganisms used in the paper. The nomenclature for bacteria should follow well-established references such as *Bergey's Manual of Determinative Bacteriology*(current edition) or the *Manual of Clinical Microbiology*, 8th ed., or validation lists and specific articles published in the *International Journal of Systematic and Evolutionary Microbiology* since January 1, 1989. Informative web sites are available as well (http://www.dsmz.de/bactnom/bactname.htm and http://www.bacterio.cict.fr).

Nomenclature and classifications of fungi and yeasts are the responsibility of the manuscript authors as guided by published sources such as *Ainsworth and Bisby's Dictionary of the Fungi*, 9th ed. (2001) and *The Yeasts: A Taxonomic Study*, 4th ed. (1998).

Names used for viruses should be those listed in the International Committee on Taxonomy of Viruses publication (*Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee of Taxonomy and Viruses, 2000*). Synonyms may be used in parentheses when the name is first used in conjunction with the approved generic (or group) and family names.

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Carta de Aceite de Publicação no periódico Journal of Infection Control

[JIC] Decisão editorial

9 mensagens

Flavia Trench <flavia.trench@gmail.com> 11 de dezembro de 2012 12:51

Para: Sr Thiago Galvão Paim <thiagopucrs@gmail.com>

Cc: Keli Cristine Reiter <kelicrisr@gmail.com>, Caio Fernando Oliveira <caioo@ufcspa.edu.br>, Pedro Alves d'Azevedo <pedro_dazevedo@yahoo.com.br>

Sr Thiago Galvão Paim,

Foi tomada uma decisão sobre o artigo submetido à revista Journal of Infection Control,
"MALDI-TOF MS performance to identify Gram-positive cocci clinical isolates in Porto Alegre, RS, Brazil.".

A decisão é:O ARTIGO FOI ACEITO PARA A PUBLICAÇÃO, tendo sido feita uma pequena sugestão pelos avaliadores relacionada abaixo.Aguardamos as modificações.

This is a comparative study of two methodologies for identifying Gram-positive cocci. This study examined the correlation of 450 strains of gram-positive cocci between two methods (MALDI-TOF MS and conventional methodology) with good agreement.

Although these data are already available in the literature, the authors

emphasize the importance of MALDI-TOF methodology in identification, fast and accuracy. Thus the work adds an another proof of this methodology and deserves to be approved for publication in this journal.

Just a comment about the author's conclusion: they mention that this methodology is inexpensive. It would be useful to compare the costs or add a reference to this conclusion in order to support it. Another alternative would let mention of cost-effectiveness only.

Flavia Trench

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Fax 45 35728177

flavia.trench@gmail.com

Journal of Infection Control

http://jic.abih.net.br/index.php/jic

Instrução aos autores para submissão de artigo científico no periódico Journal of Infection Control

Diretrizes para Autores

Journal Sections

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

- Original basic or clinical investigation (original papers);
- Brief reports of new methods or observations (brief communications);
- State-of-the-art presentations or reviews (review or mini review papers);
- Case presentation and discussion (case reports);
- Clinical infectious diseases images;
- Letters to the editor concerning previous publications;
- Editor's corner, containing ideas, hypothesis and comments (Editorial).

Original Papers

It is the most important section of the Journal. Original articles present new data about researches, issues and matters in the field of Infection Control. These articles should conform strictly to the rules of publication,containing the following sections: abstract, objective or hypothesis, experimental design and methods used (statistical data), essential features of any interventions, main outcome measures, main results of the study, discussion and conclusion. An Original Paper should contain: An abstract of no more than 300 words; No more than 7 keywords; A running title with no more than 60 characters and spaces; The text should be divided into separate sections (Introduction, Material and Methods, Results, Discussion, Conclusion, References); No more than 50 references; Number of authors should not exceed

10; Authors should state in the cover letter that the manuscript is intended to be an original paper.

Brief Communications

An abstract of no more than 200 words; No more than 4 keywords; A running title with no more than 60 characters and spaces; Text should not exceed 12 double-spaced typed pages of 23 lines each; A maximum of 2 figures or tables (or one of each); No more than 20 references; The text should not be divided into separate sections; Authors should state in the cover letter that the manuscript is intended to be a brief communication; Number of authors should not exceed 5.

Review Article

An abstract of no more than 300 words; No more than 7 keywords; A running title with no more than 60 characters and spaces; No more than 80 references; The text may be divided into sections with appropriate titles and subtitles; Number of authors should not exceed 5; Authors should state in the cover letter that the manuscript is intended to be a review or mini review article.

Case Reports

Reports of clinical cases must contain a brief introduction about the nature of the case diagnosis, whose focus is the importance of the subject. The case has to be described with data and reports of examinations, treatment and prognosis of the case, discussion about the importance of the findings and presentation of the case in relation to literature. A case report should have a special interest to the clinical research community or it has to be a rare case; or to present a new diagnostic method; or new or modified treatment. A case report article

should contain: An abstract of no more than 150 words; No more than 4 keywords; A running title with no more than 60 characters and spaces; No more than 20 references; The text may be divided into sections: brief introduction with a review of literature; case reports; and conclusion; Number of authors should not exceed 5; Authors should state in the cover letter that the manuscript is intended to be a case report article.

Clinical Infectious Diseases Images

In one doublespaced electronic document, title (no more than eight words in the title; no more than five authors may be listed, including name, highest academic degree, address, e-mail address, telephone and fax number of each author); There is no abstract; The text should be uniform and contain no more than 300 words; It could present a minimum of references (no more than 4); Number of authors should not exceed 3.

Letters to the Editor

Letters may be written in response to previous content published in The Brazilian Journal of Infection Control or on any topic ofgeneral interest or concern. In the fi rst case, the letter must emphasize the main message of the author of the article, focusing the contribution of that scientifi c article in the medical practice, drawing attention to the reference and impact it had on the community. The Letter to the Editor should contain: In one double-spaced electronic document, title and the text with no more than 23 line pages; It could contain eferences, but no more than 5; Number of authors should not exceed 5.

Editorial

The editorial containing the authors' ideas, hypotheses, points of views and comments about a relevant subject. This section is alsoused to publish tributes or notices of congress and

seminars. It should contain: In one doublespaced electronic document, title, no more than 3 authors and their affiliations, and the text with no more than 2 double-spaced pages with 23 line pages; Number of authors should not exceed 3. Manuscript Formats *Cover Letter* For all manuscripts, authors must indicate in a cover letter: Title of article; Name(s) of all author(s); Address, telephone number, fax, and e-mail of the corresponding author; A statement signed by the corresponding author confirming that the manuscript content represents the views of the coauthors, that neither the corresponding author nor the coauthors have submitted duplicate or overlapping manuscripts elsewhere, and that the items indicated as personal communications in the text are supported by the referenced person; The registration number of all randomized controlled trials and clinical trials, in accordance with the International Committee of Medical Journal Editors; In the same case, the corresponding author has to send a statement indicating that written informed consent was obtained from all participants; All original articles should indicate the number of the Ethics Committee approval. If the article does not require an approval from the Ethics Committee, the author should write it separately in the article; Animal experimentation should be carried out according to institutional guidelines for experimental use of animals; The authors should obtain written permission to reproduce fi gures and tables from other sources; If the study was supported by any institution, it has to be indicated in the cover letter. The cover letter should be in one double-spaced electronic document, title (no more than eight words in the title); no more than five authors may be listed, including name, highest academic degree, address, email address, telephone and fax number of each author; the text should contain no more than 300 words.

Supplements to the JIC include articles under a unifying theme, such as those summarizing presentations of symposia or focusing on a specific pathogenic process or antimicrobial agent. These will be added to the regular bi-monthly publication as appropriate, and will be peer

reviewed in the same manner as submitted manuscripts. For each manuscript a registration number will be assigned and the author notified that the manuscript is complete and adequate to start the review process.

Format of Articles and Letters

Contributions should be double-spaced and written in English and Portuguese (see item Translation). All manuscripts are to be typed double-spaced, including text, tables, references and legends; the character should be Time New Roman for all text, including figures, graphics and tables, or Symbol for Greek character; size 12 should be used in all manuscript. All pages are to be numbered, with the order of presentation as follows: title page, abstract, text, acknowledgements, references, tables, figure legends and figures. The manuscripts have to be saved in Word document and the figures should be saved in CorelDraw, JPG or TIF document with high resolution - minimum of 300 dpi.

Titles and Subtitles

Titles should be in bold; Subtitles should be in underscore; Titles should not have abbreviations or acronyms; Titles should not exceed two printed lines; Do not exceed 80 characters (inc. spaces).

Author Affiliations

Complete name of the authors (do not abbreviate); Affiliations of all authors, including highest degree and institution(s); Name, mailing addresses, phone and fax number, e-mail, state, city and country of the corresponding author; Acknowledgment of research grants and fellowships (agency and grant number);

Head Running

All manuscripts, except Clinical Infectious Diseases Images, Letters and Editorials have to indicate a running title; Do not exceed 60 characters (inc. spaces); Insert the head running at the top of the page.

Key Words

Consider the manuscript formats to verify the number of keywords; Use capital letter for the first word, the other should be in regular form.

Abstract

The abstract should briefly contain the objective, data, methods, results and discussion of the study or presentation to ensure the reader's understanding of the manuscript; Do not use abbreviations in abstract; Do not use references in abstract; Consider the manuscript formats to verify the number of words.

Introduction

In the text of Introduction the authors have to reveal the aim of the study, the purpose of the research, and the basic literature about the subject.

Material and Methods

This section should be subdivided by short underscore headings referring to methods used; This section cannot contain figures or tables; The material and methods used must be carefully described to allow the study repetition and to determine if the results were possible and correct; Papers with statistical testing should state the name of the test, the name for each

analysis, the comparisons of interest, a justification of that test, the alpha level for all tests, whether the tests were over two-tailes, and the actual p-value for each test; Data sets should be summarized with descriptive statistics, which should include then for each data set, a clearly labeled measure of centre (such as the mean or median), and a clearly labeled measure of variability (such as the standard deviation or range).

Results

The data of the results should be described concisely; Tables, graphics and figures have to be inserted in this section; The data presented in this section have to be oriented by universal units; Tables should be clear enough to the authors do not need the text to understand them; Tables should be presented on separate pages, portrait orientation, and upright on the page; Tables have to be a short one-line title in bold; Tables have to be numbered consecutively with Arabic numerals in the text; Symbols and abbreviations are defined immediately below the table; More information about the table should be below the symbols and abbreviations; If the table is from another source, the authors must indicate the source and send the permission to the Journal.

Figures/Graphics

Figure legend should be listed one after the other, as a part of the text document, separate from the figure files, with a short one-line title in bold. Figures should be submitted in paper or in CDR, TIF, JPG file (300 dpi) or presented in glossy photographs or as high quality laser prints on bond paper; Figures should be clear enough that the authors do not need the text to understand them; Figures should be presented on separate pages, portrait orientation, and upright on the page; Figures have to be numbered consecutively with Arabic numerals in the text; Symbols and abbreviations are defined immediately below the figure, as well as any

other informations about the figures; If the figure is from another source, the authors must indicate the source and send the permission to the Journal.

Discussion

The discussion presents the results comparing and evaluating them to literature and the existing knowledge. References to other studies should appear in the Discussion to compare the data obtained in the methods and results of the paper.

Conclusion

In this section the authors confirm or not their interpretation presented in Discussion with the existing literature. In this section the authors recommend if it is important to have more data, if the study is a innovative point of view of a subject, or if the study confirms the knowledge about the subject.

Acknowledgments

Authors can thank anyone who helped them do the work or study.

Financial Support

The authors must indicate in the cover letter if the study was supported by institutions.

Footnotes

Footnotes should be numbered consecutively and must appear superscripted and in Arabic number in the text; and their information has to be informed at the bottom of the page they appeared.

Abbreviations and symbols

All abbreviations have to be explained in the text, figure and table legends when they first appear; Include the abbreviation in parenthesis after they first appearance. Do not abbreviate units [(5 mL, not 5 milliliters (mL)];Do not abbreviate institutions;Abbreviations must follow the format of the National Library of Medicine (USA) as in Index Medicus. Units Follow the use of the The Système International (SI) (http://physics.nist.gov/cuu/Units).

References

They should go in the final part of the article, according to the quotation order in the text, in which should appear the Arabic numerals superscripted. Please quote all the authors in works with until six authors; after six authors, quote the first three followed by the expression et al. Reference Manager or Endnote programs are strongly recommended for use adopting the "Vancouver" style. Examples for reference citation are presented below. Authors should consult NLM's Citing Medicine for additional information on the reference formats.

Article

Smith JC, Charles RS. Microbes and water filters. Journal of Water Purification 1996; 20:165-70.

Yang CW, Chen JC et al. Disproportional exaggerated arpartate transaminase is a useful prognostic parameter in late leptospirosis. World J Gastroenterol 2005; 11:5553-6.

Book Chapter

Taylor DM, Personnet J. Epidemiology and natural history of Helicobacter pylori infection. In: Blaser MJ, Smith PD, Ravdin J eds. Infections of the gastrointestinal tract. New York: Raven Press, 1994.

Book

Polak JM, Van Noordan S. An introduction to immunochemistry: current techniques and problems. Oxford, UK: Oxford University Press, 1987.

Abstract

Blatt SP, Butzin CA, Lucey DR, Melcher GP, Hendrix CR. Anergy status and CD4 CD29 memory T-cells predict progression to AIDS (abstract PoB 3480). In: Program and abstracts: VIII International Conference on AIDS (Amsterdam). Amsterdam: CONGREX Holland, 1992.

SCOPE AND POLICY

The aim of The Journal of Infectious Control (JIC) is to be relevant in the broadest sense to all aspects of Infectious Control and its fields. The manuscripts submitted to JIC should develop new concepts or experimental approaches; they have to describe new principles or improvement of an existing method and their results; they have to bring new data about a subject which will be important to physicians; so they could not be a single presentation of known data.

TRANSLATION

The translation costs will have to be paid by the authors. After the review process, when the authors are notified that a manuscript is accepted, authors will be asked to arrange for the translation, or if it is done by the JIC, the cost for translation will be billed along with instructions for payment. The JIC will continue to publish papers submitted in English at no additional cost.

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All the listed authors have to agree on all contents (authors must sign the agreement upon receiving it with the article galley proof) and they are responsible for all informations included in the text. The corresponding author is responsible for all communications between the journal and all coauthors, before and after publication. The corresponding author has to make a statement confirming that the content of the manuscript represents the views of the coauthors, that neither the corresponding author nor the coauthors have submitted duplicate or overlapping manuscripts elsewhere, and that the items indicated as personal communications in the text are supported by the referenced person. Any changes to the author list after submission, such as a change in the order of the authors, or the deletion or addition of authors, needs to be approved by a signed letter from every author. The editors of JIC may seek advice about submitted papers not only from technical reviewers but also on any aspect of a paper that raises concerns. These may include, for example, ethical issues or issues of data or materials access. The authors warrant that the manuscript is original and contains no matter which is defamatory or is otherwise unlawful or which invades individual privacy or infringes any proprietary right or any statutory copyright.

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exclude from the publication, and the authors will be accountable for the plagiarism.

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The Journal of Infection Control (JIC) is a peer-review Journal, so all papers (except Letters to the Editor) are evaluated by this system. If the paper follows the scope of the Journal, it will be sent to 2 or 3independent reviewers, selected by the editors (2 from the editorial board and 1 any other expertise). Authors may suggest appropriate consultants for review of the

manuscript, but these suggestions may not be followed.

Timing

The review process will ordinarily require one month.

Instrução aos autores para submissão de artigo científico no periódico *Journal of Medical Microbiology*

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2.2 Pre-submission checklist

Authors must:

- read the Information for Authors and ensure that their paper complies with this before submission;
- all agree to the submission and agree that the corresponding author may act on their behalf throughout the review and publication process;
- provide the names and contact details of at least three (and not more than five) potential reviewers;
- obtain permission for any citations of personal communications or unpublished results; this should be confirmed in a covering message;
- indicate the Contents Category for the paper on the title page (it should also be entered in the Contents Category field of the online submission form in Bench>Press);
- use continuous line numbering throughout the manuscript, to facilitate online reviewing;

- ensure that citations of references in the text and references list conform to journal style;
- upload any supplementary material associated with the paper as a supplementary file(s) for peer review with the paper;
- upload any cited papers that have been accepted for publication but are not yet published as a supplementary file(s);
- include an accession number from one of the public databases (GenBank, EMBL, DDBJ or PIR) if the paper reports new sequence data; the relevant deposition criteria for the database must be adhered to.

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2.3 Preparing files for submission

Papers can be submitted initially either as a single PDF file or as separate word-processor and image files, which will be compiled into a PDF by the system. The submission system will also attempt to prepare an HTML version of the reference list with links to papers that it can identify on the PubMed and HighWire databases, for use by the reviewers. This conversion will not work reliably if the reference format is incorrect (see the the section on References for the correct style); however, an HTML reference list is not essential for review of the paper to proceed. Supplementary material should be submitted as a separate file(s), rather than being incorporated within the single PDF or word-processor file. 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.

Submission as a single PDF. Please refer to the Help pages on the submission site for guidelines on preparing PDFs for submission, including advice on reducing the size of image files (the submitted PDF should preferably not be much larger than 1 MB).

Submission as separate word-processor and image files. Most standard word-processor files (including .docx files produced in Word 2007 or 2010) will convert successfully to PDF. Times, Times New Roman, Courier, Helvetica and Arial, and the Symbol font for special characters, are the recommended fonts. Other fonts are not guaranteed to convert successfully convert to PDF. Tables for the main paper must be prepared as part of the word-processor file; they must not be supplied as images or Excel files. (Excel files are, however, acceptable for supplementary data). Word-processor files including inserted image files will normally be converted successfully to PDF by the system, but please note that files using OLE (Object Linking and Embedding) technology to display information or embedded files are not supported. If the conversion is not satisfactory, either convert the file to PDF yourself, and submit that, or submit the image files separately.

The file types that are supported for submission as separate image files for conversion to PDF are PDF, GIF, TIFF, EPS, JPEG and PPT. A resolution of 300 d.p.i. at a reasonable size of reproduction is recommended; in other words, 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. The following file types are not supported at the initial submission stage as they cannot be converted to PDF by the system: bitmap (.bmp), PICT (.pict), Excel (.xls), Photoshop (.psd), Canvas (.cnv), CorelDRAW (.cdr) and locked or encrypted PDFs. Image files will be converted to PDF and added to the end of the manuscript PDF produced by the system. If any of the image files are very large, it is advisable to reduce their size before submission if possible: refer to the Help with Online Submission pages for guidelines on how to do this.

Our requirements for files intended for publication are different from those for files that will be converted to PDF by the Bench>Press system as part of an initial submission, as set out in the Files for Publication section of these instructions. If you are unsure whether your file formats are suitable, please contact the Editorial Office.

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2.4 General style and layout

2.4.1 Layout

The paper must be written in clear and concise English, normally in the past tense. All papers should normally include: Title page; Summary (Abstract) (not required for Editorials and Correspondence); Acknowledgements; References; Tables; and Figures, with legends. The body of Full papers should be divided into Introduction; Methods; Results; and Discussion. It is often appropriate to combine the Results and Discussion. Figures and tables should only be used to illustrate points that cannot easily be described in the text.

Authors should consult a recent issue of the journal for the layout of headings, tables, etc. Guidance on the presentation of individual sections is given below.

2.4.2 Title page

This should carry the following information.

The title of the paper. The title should provide a concise statement of the contents of the paper.
A short 'running title', of not more than 55 characters (including spaces), for use as a headline.
The Contents Category for the paper.
The names of the authors. Author names should be given in upper- and lower-case, not in all

capitals, to avoid ambiguities such as 'van' and 'Van'. The author for correspondence must be clearly indicated. It is permissible to include the names of more than one author as corresponding author, but a single author must act as the point of communication during the peer review process.

The name and address of the laboratory or laboratories where the work was done, and present addresses of authors who have since moved.

An email address and telephone and fax numbers for the corresponding author.

A footnote 'The GenBank[/EMBL/DDBJ] accession number for the [16S rRNA gene/gyrA, etc.] sequence of XXXXX is XX00000', where a new sequence(s) has been determined.

If appropriate, a footnote defining any non-standard abbreviations. Guidance on abbreviations not requiring definition is given in the Abbreviations section.

2.4.3 Summary (Abstract)

This section is likely to be read by more people than the full paper, and many abstracting services use authors' summaries without modification. It is therefore important that this section is clear and comprehensible in its own right. References should not be cited, and any non-standard abbreviations used must be defined.

2.4.4 Introduction

This should state the objectives of the work, but should not contain a detailed summary of the results. Authors should not assume that all readers will know why an area is worth studying; they should briefly make this clear. Previous relevant work should be sufficiently cited but this should not constitute a full review.

2.4.5 Methods

Sufficient detail should be provided to allow the work to be repeated. The suppliers of

chemicals and equipment should be indicated if this may affect the results. Suppliers' addresses should not be given unless this is considered essential for a particular reason.

2.4.6 Results

There should be sufficient subheadings to make clear how the work was organized, what the key questions being addressed were, how one experiment led to another, and perhaps what conclusions were reached. A reader should gain a clear picture of the work from the subheadings.

Reproducibility of results should be indicated. It should be stated how many times an experiment was repeated and whether means or representative results are shown. Variability should be indicated statistically wherever possible; when error terms are given, the measure of dispersion and the number of observations should be stated. Statistical techniques used must be specified, and where necessary they should be described fully or a reference given. If results are expressed as percentages, the absolute value corresponding to 100% should be stated.

2.4.7 Discussion

This should not recapitulate the results, and should not be too long. Excessive discussion of few facts often gives an impression of poor science. Subheadings should be used where appropriate, to highlight the points under discussion. It may be helpful to list the main conclusions at the end. A combined Results and Discussion section is encouraged where appropriate.

2.4.8 Acknowledgements

An Acknowledgements section is not compulsory but may be included. If required, please state the names of funding bodies and grant numbers in this section. Authors may also wish to acknowledge individuals who have contributed materials, expertise or time to the study who are not named as authors.

2.4.9 References

References in the text should be cited as follows: two authors, Smith & Jones (1996) or (Smith & Jones, 1996); three or more authors, Smith et al. (1996) or (Smith et al., 1996). References to papers by the same author(s) in the same year should be distinguished in the text and the reference list by the letters a, b, etc. (e.g. 1996a or 1996a, b).

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.

2.4.10 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.

2.4.11 Figures

This section outlines journal policy on figures. See these links for advice on preparing figures for inclusion as a PDF for submission 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.

2.4.12 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.

2.4.13 Supplementary material

Material associated with a paper but not suitable for print publication (e.g. large datasets, sequence alignments, 3D structures, 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 sometimes suggest that figures or tables that the author has 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. Supplementary figures and tables should be named Fig. S1, Table S1, etc., and be cited accordingly in the main paper. A heading and, if appropriate, a short legend or text description must be supplied for each supplementary data item.

File types and formatting for supplementary data. The contents of the supplementary file should be indicated in the 'File label' field when the file is uploaded. Most file types can be supported but authors should try to avoid files that require unusual software, because these will be of limited use to readers. Very large files should also be avoided where possible because they may be difficult to download. Editorial staff may apply stylistic editing to supplementary files, and will, where possible, convert the files to PDF format for online publication.

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 (see http://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. EcoRI not EcoRI, etc.; HindIII not HindIII, HindIII, Hind III, etc.

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3.4 Genetic nomenclature

For bacterial gene names, use e.g. gyrA not gyrA; arg-1 not arg1 or arg1, 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; ED50; 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; LD50; 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.; Pi, PPi; 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 = 3.7×1010 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

Mr (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 Mr 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×Mr.

3.6.3 Absorbance, optical density and attenuance

The term absorbance, A, should be used for the quantity log(I0/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. A280, OD600).

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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'.