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Avaliação da Capacidade de Formação de Biofilme, Susceptibilidade aos antimicrobianos e Detecção de Fatores de Virulência em Isolados Clínicos de *Enterococcus* spp.

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“Dedico essa Dissertação
à minha mãe,
razão da minha vida”

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mas Graças a Deus,
não sou mais o que era antes”.
(Marthin Luther King)

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LISTA DE ABREVIATURAS E SIGLAS

Agg (AS) = Substância de agregação (*aggregation substance*)

BMD = Microdiluição de caldo (*Broth Microdilution Method*)

CLSI = (*Clinical Laboratory Standards Institute*)

CHSCMPA = Complexo Hospitalar Santa Casa de Misericórdia de Porto Alegre

DNA = Ácido desoxirribonucléico (*deoxyribonucleic acid*)

DNSE = Enterococos não-suscpetíveis à daptomicina (*Daptomycin nonsusceptible enterococci*)

Esp = Proteína de Superfície de Enterococos (*Enterococcal Surface Protein*)

GelE = gelatinase

HLAR = Altos níveis de resistência aos aminoglicosídeos (*High-level aminoglycoside resistance*)

UTI = Unidade de Terapia Intensiva *(Intensive Care Unit)*

IAAS = Infecções Associadas à Assistência à Saúde

MDR = Resistente a múltiplas drogas (*Multidrug-resistant)*

MIC = Concentração inibitória mínima (*minimal inhibitory concentration*)

MW = marcador de peso molecular (*molecular weight)*

PCR = Reação em cadeia da polimerase (*polimerase chain reaction*)

PBP = proteína de ligação à penicilina

PFGE = Eletroforese em gel de campo pulsado (*Pulsed-Field Gel Electrophoresis*)

UFCSPA = Universidade Federal de Ciências da Saúde de Porto Alegre

VRE = Enterococos resistentes à vancomicina (*Vancomycin resistant enterococci*)

VRE_{fm} = *Enterococcus faecium* resistente à vancomicina (*Vancomycin resistant Enterococcus faecium*)

UPGMA = *Unweighted pair group method using arithmetic averages*

ITU = Infecção do trato urinário (*Urinary Tract Infection*)

RESUMO

Enterococcus faecalis e *Enterococcus faecium* estão entre os principais agentes etiológicos de infecções associadas à assistência à saúde (IAAS). Tais espécies podem apresentar, além de perfi de multiresistência, decorrentes da aquisição de elementos genéticos móveis, a capacidade de formar biofilmes que funcionam como barreira à ação dos agentes antimicrobianos. Genes como os da gelatinase (*gelE),* da proteína de superfície de *Enterococos (esp)* e da substância de agregação (*agg)*, além de terem seus produtos associados ao processo de adesão, têm sido associados ao processo multifatorial de formação do biofilme. O surgimento da resistência adquirida a antibióticos sistêmicos que antes eram eficazes no tratamento de infecções enterocócicas também se tornaram uma preocupação crescente. Neste contexto, o objetivo do presente estudo foi avaliar a capacidade de produção de biofilme em isolados de *Enterococcus* spp. obtidos a partir de amostras clínicas de pacientes internados no Complexo Hospitalar Santa Casa de Misericórdia de Porto Alegre (CHSCMPA), detectar a presença de genes de virulência e de um de seus produtos (gelatinase) e avaliar o perfil de susceptibilidade desses micro-organismos a diferentes antimicrobianos. Foram realizadas coletas consecutivas de amostras de *Enterococcus* spp. no período de setembro de 2012 a agosto de 2013. O gênero foi caracterizado por métodos fenotípicos convencionais e a identificação das espécies e dos fatores de virulência foi obtida mediante técnica de PCR. Foram realizadas metodologias para caracterização dos perfis de susceptibilidade, indução da formação do biofilme e análise de clonalidade das amostras de *E. faecium* resistentes à vancomicina (VRE_{fm}). Após esta etapa, o trabalho foi dividido em dois segmentos. O primeiro utilizou todas as amostras que foram coletadas nos seis meses iniciais do estudo. Dos 240 isolados de *Enterococcus* spp., 221 (92,1%) foram identificados como *E. faecalis* e 19 (7,9%) como *E. faecium*. A espécie *E. faecalis* apresentou uma alta habilidade para formação de biofilme e a presença dos fatores de virulência foi significativa. Em contrapartida, a resistência aos antimicrobianos foi maior entre os isolados de *E. faecium.* O segundo segmento utilizou somente as amostras caracterizadas como *Enterococcus faecium* resistentes à vancomicina e que foram obtidas ao longo dos 12 meses do estudo. Todos os 27 isolados de VRE_{fm} carreavam o gene *vanA*, e apresentaram resistência a ampicilina e ciprofloxacina. Também foi observada em 18,5% dos isolados a não-susceptibilidade a daptomicina. Na análise da diversidade genética foi observada um perfil clonal em 88,9% dos isolados. Os resultados encontrados no nosso estudo contribuem para a compreensão do perfil de susceptibilidade, da virulência e das características moleculares de *Enterococcus* circulantes na cidade de Porto Alegre, auxiliando na compreensão da patogenicidade desses micro-organismos e na implantação de medidas que previnam a disseminação das infecções.

Palavras-chave: *Enterococcus faecalis, Enterococcus faecium,* biofilme, fatores de virulência, resistência a vancomicina, resistência a daptomicina.

ABSTRACT

Enterococcus faecalis and *Enterococcus faecium* are among the main species causing Healthcare-associated infections (HAI). Such species may have acquired resistance in addition to some antibiotics, the ability to form biofilms which act as a barrier to the action of antimicrobial agents. Genes such as gelatinase (*gelE*), of enterococci surface protein (*esp*) and aggregation substance (*agg*) have been associated with multifactorial process of biofilm formation. The emergence of acquired resistance to systemic antibiotics that were effective in treating enterococcal infections have also become an increasing concern. In this context, the aim of this study was to evaluate the ability of biofilm production in isolates of *Enterococcus* spp., obtained from clinical specimens of patients hospitalized in a Hospital Santa Casa de Porto Alegre, detect the presence of virulence genes and assess the susceptibility of these organisms to different antibiotics. Were collected consecutive strains of *Enterococcus* spp. from September 2012 to August 2013. The genus was characterized by phenotypic methods and identification of species and virulence factors was obtained by PCR. Methods for characterizing the susceptibility profiles, inducing the formation of biofilm and clonality analysis of samples of Vancomycin Resistant *E. faecium* (VRE_{fm}) were performed. After this step, the work was divided into two segments. The first used all the samples that were collected in the first six months of the study. Of the 240 isolates of *Enterococcus* spp., 221 (92.1 %) were identified as *E. faecalis* and 19 (7.9%) and *E. faecium*. The species *E. faecalis* showed a high ability for biofilm formation and the presence of virulence factors was significant. In contrast, the antimicrobial resistance was higher among isolates of *E. faecium*. The second segment used only samples identified as *E. faecium* with high levels of resistance to vancomycin, which were obtained over the 12 month study. All 27 isolates carried the gene *vanA* , and were resistant to ampicillin and ciprofloxacin. It has also been observed in 18.5 % of isolates non-susceptibility to daptomycin. In clonal analysis we observed a high similarity among 88.9 % of the isolates. The results of our study contribute to the understanding of the susceptibility profile and molecular characteristics of *Enterococcus* circulating in the city of Porto Alegre, assisting in the implementation of measures to prevent the spread of infections.

Key words: *Enterococcus faecalis, Enterococcus faecium,* biofilm, virulence factors, vacomycin resistance, daptomycin resistance.

SUMÁRIO

1. INTRODUÇÃO

1.1 *Enterococcus* spp.

Enterococos são habitantes naturais da cavidade oral, microbiota intestinal normal e trato genital feminino de humanos e animais. Normalmente enterococos coexistem com o hospedeiro, porém na era da medicina atual, fatores como antibióticos de amplo-espectro e imunosupressão do hospedeiro, podem alterar o papel deste micro-organismo, de comensal para patógeno oportunista, rompendo esse comensalismo até então equilibrado (Gilmore *et al.,* 2002; Teixeira *et al.,* 2011).

Portanto, há mais de 100 anos, desde o surgimento do primeiro caso de endocardite aguda causada por esse micro-organismo, enterococos são considerados agentes causadores de Infecções Associadas à Assistência à Saúde (IAAS). As infecções do trato urinário (ITUs) são mais comumente associadas aos enterococos. Frequentemente, também são isolados em infecções intra-abdominais, pélvicas e de tecidos moles, porém nesses casos, raramente são a causa de infecções monomicrobianas nesses sítios (Richards *et al.*, 2000 Gilmore *et al.,* 2002; Orsi & Ciorba, 2013). Enterococos também são a terceira causa de bacteremia nosocomial e endocardite, que por sua vez é uma das mais sérias infecções causadas por este micro-organismo. Raramente, causam osteomelites, artrites sépticas e pneumonia (Malani *et al.,* 2002; Chuang-Smith *et al.,* 2010) como também são raros os casos que acometem sistema nervoso central (Iaria *et al.,* 2005; Jones *et al.,* 2007; Khan & Elshafi, 2011; Daubié *et al.,* 2013) e trato respiratório (Umgelter *et al.,* 2007; Carabalona *et al.,* 2013).

Nos anos 70 e 80, a ocorrência de resistência aos antimicrobianos levou à emergência desse gênero, passando a ser vistos com um grande desafio no

ambiente hospitalar (Arias & Murray, 2012). Os fatores de risco para infecção por enterococos são principalmente idade avançada, hospitalização, presença de cateter, terapia com múltiplos antimicrobianos e imunossupressão (Di Rosa *et al*., 2006).

1.1.1 Taxonomia

Durante muitos anos, enterococos foram classificados como estreptococos do grupo D de Lancefield. Em 1984, Schleifer & Klipper-Bälz (1984) propuseram a criação do gênero *Enterococcus*, após estudos de hibridização de DNA-DNA e sequenciamento da subunidade 16S rRNA que revelaram uma distância genética com *Streptococcus.* As primeiras espécies caracterizadas no gênero foram *Enterococcus faecalis* e *Enterococcus faecium.*

Até os dias atuais, mais de 50 espécies de *Enterococcus* já foram descritas (Euzéby, 2013), e podem ser encontradas e identificadas em uma diversidade de hospedeiros como mamíferos, pássaros, répteis, insetos e disseminados no solo, plantas e água como contaminantes fecais, devido a sua capacidade de sobreviver em condições adversas (Facklan *et al.*, 2002).

1.1.2 Características

Enterococcus spp. são organismos pertencentes ao grupo de bactérias ácido-láticas (LAB), Gram-positivas, anaeróbias facultativas, não possuem enzimas do citocromo, são catalase negativas e não formadoras de esporos. Apresentam-se em forma de cocos em pares ou cadeias curtas, ou como células únicas. Crescem em temperatura variando entre 10ºC e 45ºC e sobrevivem a 60ºC por 30 minutos. Toleram variações de pH e crescem em meio contendo 6,5% de cloreto de sódio e

40% de sais biliares. Produzem a enzima leucina-aminopeptidase (LAP) e a maioria das espécies hidrolisam o substrato pirrolidonil-β-naftilamida (PYR) (Murray *et al.*, 1990; Foulquié-Moreno *et al.*, 2006).

As principais espécies causadoras de infecção no homem são *Enterococcus faecalis* (85 a 90% dos casos) e *Enterococcus faecium* (5 a 10% dos casos) (Tavares, 2000; Kayaoglu *et al*, 2004). Entretanto, a proporção de isolamento dessas duas espécies pode variar, principalmente quando se trata de surtos hospitalares, onde *E. faecium* se sobressai, uma vez que esse micro-organismo apresenta uma notável capacidade de colonizar indivíduos sadios e doentes e persistir no hospedeiro, podendo se disseminar com maior facilidade entre diferentes indivíduos (Cattoir & Leclercq, 2013). Esta mudança na epidemiologia de enterococos tem sido atribuída à disseminação de uma linhagem genética de *E. faecium* designada como CC17, que apresenta elevados níveis de resistência à maioria dos antibióticos utilizados na clínica para o tratamento das infecções enterocócicas associada a presença de fatores de virulência que conferem à essas cepas incrível adaptação ao ambiente nosocomial (Panesso *et al.,* 2010).

A espécie *E. faecalis* é conhecida por ser mais virulenta, e como ocorrem em maior número de infecções do que *E. faecium*, sugere-se que ela seja amplamente mais adaptada ao hospedeiro devido a relação comensal que possuem (Gilmore *et al.,* 2002). *Enterococcus faecium* tem emergido como um dos principais patógenos multirresistentes no ambiente hospitalar devido à sua plasticidade genômica para adquirir e disseminar elementos genéticos móveis associados a resistência bacteriana (Palmer *et al,* 2011; Lebreton *et al.,* 2013).

Murdoch *et al.,* (2002) realizou um estudo com 1785 isolados de enterococos de corrente sanguínea obtidos em um período de 25 anos e demonstrou que,

embora o *E. faecalis* tenha sido sempre mais prevalente que *E. faecium*, com o passar dos anos, o número de *E. faecium* isolados aumentou. Esse aumento na frequência de *E. faecium* isolados pode estar relacionado com uma maior imunossupressão dos pacientes, tornando-os mais propensos à infecções, bem como ao avanço nas técnicas de identificação das espécies (Kurup *et al.,* 2008).

Casos de isolamento de *E. gallinarum, E. casseliflavus, E.hirae, E. raffinosus*, *E. avium* e *E. durans* como causa de infecções em humanos são menos frequentes, porém tem sido relatados (Mondino *et al.,* 2003; d'Azevedo *et al.,* 2004, 2006; Titze-deAlmeida *et al.,* 2004; Jasovich *et al.,* 2008; Bender *et al.,* 2010; Shirano *et al.,* 2011; Anghinah *et al.,* 2013).

1.2 Fatores de Virulência

Apesar do potencial patogênico, enterococos exibem baixos níveis de virulência, uma vez que são colonizadores naturais do trato gastrointestinal de humanos. Considerando esse baixo potencial de virulência, podem ser utilizados como probióticos em humanos e animais. Pensava-se que a ascensão de enterococcos no ambiente hospitalar se devia apenas a resistência aos antimicrobianos que lhe dava certa vantagem seletiva, porém, hoje se discute o fato destes micro-organismos apresentarem diferentes genes codificadores de proteínas que podem desempenhar um papel fundamental na patogenicidade do enterococo, com foco especial nas moléculas de adesão (Arias & Murray, 2012). A virulência dos enterococos é multifatorial, com a participação de diferentes fatores como a habilidade para colonização, e, de forma especial, a capacidade de aderir a uma série de proteínas de matriz extracelular e as células epiteliais (Fisher & Phillips, 2009).

Há uma grande preocupação sobre o potencial dos enterococos em causar infecções graves. O processo de invasão ocorre inicialmente por danos teciduais ao hospedeiro que facilitam sua entrada, e a presença de elementos patogênicos do micro-organismo que vão auxiliar no seu avanço e proliferação (Sharifi *et al.,* 2012).

Vários fatores já foram identificados nesse gênero e acredita-se que os mesmos possam desempenhar um papel crucial na persistência desse patógeno nos mais diversos sítios anatômicos (Kafil *et al.*, 2013). Em *E. faecalis,* fatores como gelatinase (GelE), substância de agregação (AS) e a proteína de superfície de Enterococos (Esp) são considerados como possíveis fatores de virulência associados essencialmente ao processo de adesão e com potencial participação no processo de formação do biofilme (Leavis *et al.*, 2004; Furumura *et al.,* 2006,

Chuang-Smith *et al.*, 2010). A formação do biofilme, por sua vez é um mecanismo clássico de virulência, que será descrito com maiores detalhes abaixo.

1.2.1 Gelatinase (GelE)

Gelatinase é uma protease cuja função é proporcionar aos micro-organismos nutrientes peptídicos. O gene *gelE* codifica essa metaloprotease que pode causar danos ao hospedeiro, uma vez que é capaz de hidrolisar gelatina, colágeno, caseína e hemoglobina (Giridhara Upadhyaya *et al.,* 2009).

A presença do gene *gelE* não indica necessariamente atividade da enzima gelatinase. Em muitos casos, os genes estão silenciosos e poderão ser ativados a qualquer momento sob algum fator ambiental (Fracalanzza *et al.,* 2007).

Essa enzima também tem sido relatada como envolvida no processo de formação do biofilme, através de sinalização do tipo *quorum-sensing* (Hancock e Perego, 2004; Lu *et al.,* 2013).

1.2.2 Proteína de Superfície de Enterococos (Esp)

A proteína de superfície de *Enterococcus* (Esp) é uma adesina codificada pelo gene *esp,* que se encontra na superfície da parede celular, contribuindo para a colonização e persistência do patógeno. Algumas modificações estruturais nessa proteína podem impedir o reconhecimento desse micro-organismo pelo sistema imune do hospedeiro, favorecendo sua permanência no sítio da infecção (Shankar *et al.,* 1999). Alguns estudos relataram que essa proteína também pode ser responsável pela formação de biofilme por estes micro-organismos (Toledo-Arana *et al.,* 2001; Creti *et al*., 2006).

Além disso, Esp tem mostrado um papel importante na adesão bacteriana às células do trato urinário, também contribui para a colonização dessa espécie em válvulas cardíacas (Gilmore *et al.,* 2002; Tendolkar *et al.,* 2004; Heikens *et al.,* 2011) e parece ser uma característica importante de algumas linhagens de *E. faecium* (variante esp_{fm}) extremamente bem adaptadas ao ambiente hospitalar (Baldassarri *et al*., 2001).

1.2.3 Substância de Agregação

A substância de agregação é uma glicoproteína de superfície codificada por plasmídeos e expressa em resposta a indução por feromônios, que auxilia na transferência de material genético durante a conjugação e na adesão do enterococo à superfície de diferentes células eucarióticas. Foi observado que em *E. faecalis,* a substância de agregação forma grandes agregados celulares *in vivo* que podem contribuir para a patogênese da infecção, aumentando a hidrofobicidade da superfície celular e a vegetação em encocardite experimental (Foulquié-Moreno *et al.,* 2006; Fisher *&* Phillips, 2009; Schlievert *et al.,* 2010).

1.2.4 Biofilme

Biofilmes são definidos como associações de células bacterianas aderidas em superfícies bióticas ou abióticas, envolvidas em uma densa matriz exopolissacarídica produzida pelas próprias bactérias, e que exibe uma alteração fenotípica considerando sua razão de crescimento e transcrição genética (Costerton *et al.*, 1995; Costerton *et al.*, 1997; Donlan & Costerton, 2002).

Para que ocorra a formação do biofilme, primeiramente, as células planctônicas se aderem reversivelmente a uma superfície, logo após a adesão se

torna irreversível, e posteriormente ocorre a etapa de crescimento e divisão celular com a ativação de genes responsáveis pela produção de polissacarídeo, e por fim o desenvolvimento adicional que forma o biofilme maduro, tenaz e quase impenetrável. Os fatores que influenciam a produção do biofilme envolvem um meio de crescimento rico em nutrientes como a glicose, disponibilidade de ferro e CO_2, osmolaridade, pH e temperatura (Pillai *et al*., 2004).

Os micro-organismos associados aos biofilmes são considerados um importante desafio nas instituições de saúde, pois possuem a capacidade de funcionar como barreira à ação dos antimicrobianos (Donlan *et al.*, 2001). Sua formação é uma estratégia vital adotada pelas células para sobrevivência sob condições adversas (Sepandj *et al*., 2003) e proteção contra a ação do sistema imunológico do hospedeiro. Estima-se que os biofilmes estejam envolvidos em 65% das infecções bacterianas no homem (Nascimento e Taveira, 2001).

Em biofilmes, os enterococos são altamente mais resistentes a antibióticos (10 a 1000 vezes) que enterococos de crescimento planctônico, uma vez que formam uma barreira mecânica que impede a passagem do antimicrobiano. Dessa maneira, o impacto da formação do biofilme nas infecções enterocócicas é cada vez maior e os mecanismos e fatores envolvidos tem se tornado cada vez mais alvo de estudos (Mohamed & Huang, 2007; Garsin & Willems, 2010).

1.3 Resistência aos antimicrobianos

Um fator que foi importante para o reconhecimento dos enterococos como importante patógeno nosocomial é a sua resistência intrínseca e adquirida a diferentes antimicrobianos, o que contribui para sua adaptação em ambientes adversos e favorece a colonização do trato gastrointestinal, além de posterior transmissão entre pacientes (Gilmore *et al.,* 2013).

Essa resistência pode surgir por mutações na sequência de bases do DNA, ou ser adquirida pela transferência de genes de outros micro-organismos. Em geral, a resistência envolve transformação e conjução, principalmente pela aquisição de genes determinantes de resistência, em plasmídios e transposons, por intermédio de outros micro-organismos portadores do fenótipo (Tavares, 2000).

Enterococos apresentam resistência intrínseca, mediada por genes cromossomais, a cefalosporinas, sulfonamidas, clindamicina e baixos níveis de β-lactâmicos e aminoglicosídeos. A resistência adquirida ocorre a virtualmente todas as classes de antimicrobianos: macrolídeos, cloranfenicol, tetraciclina, fluoroquinolonas e glicopeptídeos, além de níveis elevados de resistência a aminoglicosídeos e β-lactâmicos (Shepard & Gilmore, 2002).

O tratamento das infecções enterocócias é, algumas vezes, desafiador. A combinação da gentamicina em doses elevadas com penicilina ou ampicilina, durante pelo menos quatro semanas, são os fármacos de escolha para infecções enterocócicas sistêmicas, como endocardite e bacteremia. Quando existe resistência a níveis elevados de aminoglicosídeos ou ampicilina, as drogas de escolha são os glicopeptídeos (vancomicina ou teicoplanina), embora as taxas de erradicação sejam menores. No caso de enterococos resistentes à vancomicina, o tratamento dependerá do antibiograma: podem ser utilizadas linezolida ou daptomicina e, no

caso de *E. faecium,* também pode ser administrado quinupristina/dalfopristina (Landman & Quale, 1997; Shepard & Gilmore, 2002; Arias & Murray, 2012).

Hoje, a maioria dos *E. faecium* recuperados de infecções são resistentes à ampicilina, vancomicina e apresentam altos níveis de resistência aos aminoglicosídeos, o que afeta diretamente a efetividade do sinergismo entre os antibióticos (Arias & Murray, 2012).

A resistência aos β-lactâmicos é associada com a superprodução de uma proteína de ligação à penicilina (PBP) de baixa afinidade ou a produção de enzimas β-lactamases. A resistência à ampicilina é rara em *E.* faecalis, porém em *E. faecium* ocorre em 90% dos isolados, e está associada a produção de PBP5, umas das 5 PBPs existentes em enterococos, que confere resistência a todos os β-lactâmicos (Fontana *et al.,* 1996; Arias & Murray, 2012).

Como a resistência aos aminoglicosídeos é instrínseca em enterococos, esse antimicrobiano só apresenta um efeito bactericida sinérgico quando utilizado em associação com β-lactâmicos ou glicopeptídeos. Alterações do sítio-alvo no ribossomo e ineficiência no transporte do antimicrobiano através da membrana citoplasmática são mecanismos que promovem a resistência a baixos níveis em enterococos. A resistência adquirida a altas doses ocorre devido à produção de enzimas modificadoras de aminoglicosídeos (fosfotransferases, nucleotidiltrasnferases e acetiltransferases) que levam a perda do sinergismo e diminuem o efeito bactericida no tratamento de infecções por enterococos (Shepard & Gilmore, 2002).

Ciprofloxacina, antibiótico do grupo das fluoroquinolonas, é utilizado para o tratamento de ITUs. A resistência a ciprofloxacina ocorre devido a mutações nos

genes *gyrA* (que codifica a subunidade A da DNA-girase) e no gene *parC* (que codifica a subunidade da topoisomerase IV) (Kanematsu *et al.,* 1998).

A resistência a Linezolida, antibiótico pertencente ao grupo das oxazolidinonas, é associada com a presença de mutações no gene do RNA ribossomal 23S (Prystowsky *et al.,* 2001; Patel *et al.,* 2013).

A quinupristina/dalfopristina é a combinação de duas estreptograminas (B e A) com ativade contra *E. faecium*, porém não apresentam atividade contra *E. faecalis* que é intrinsicamente resistente devido a expressão do gene *isa*. A resistência a quinupristina é mediada por determinantes de resistência (por exemplo, *ermB)* que codifica uma metilase ribossômica impedindo a ligação do fármaco a seu alvo, ou por *vgb* que codificam lactonases que inativam as estreptograminas do tipo B. Já a resistência a dalfopristina, é mediada por genes codificadores de acetiltransferases (*satA e satG*) que inativam as estreptograminas do tipo A. A resistência a combinação dos dois antimicrobianos está sempre associada a um gene de resistência para estreptograminas do tipo A (Bozdogan & Leclercq, 1999; Soltani *et al.,* 2000).

1.3.1 *Enterococcus* Resistentes à Vancomicina (VRE)

Vancomicina é um antimicrobiano pertencente à classe dos glicopeptídeos. Seu mecanismo de ação visa inibir a síntese da parede celular bacteriana. Os fatores de risco para o desenvolvimento de uma infecção por VRE são: proximidade física com pacientes infectados ou colonizados; longo período de hospitalização; tratamento com múltiplos antimicrobianos; internação em instituições de longa permanência, unidades cirúrgicas ou UTIs; transplantes de órgãos sólidos e medula;

co-morbidades como diabetes, falência renal ou hemodiálise, e presença de cateter urinário (Sydnor & Perl, 2011; Arias & Murray, 2012; Shorman & Al-Tawfiq, 2013).

A resistência ocorre devido a uma alteração no alvo de ligação D-alanil-D-alanina por para D-alanil-D-lactato (que produz níveis elevados de resistência) ou D-alanil-D-serina (que produz níveis baixos de resistência), diminuindo a afinidade pela molécula de vancomicina e prejudicando a ação antimicrobiana desta droga. Foram descritos até o momento nove genes em enterococos que codificam diferentes fenótipos de resistência a glicopeptídeos (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* e *vanN*), sendo o genótipo *vanA* (caracterizado por altos níveis de resistência a vancomicina e teicoplanina) e *vanB* (caracterizado por níveis variados de resistência a vancomicina e susceptibilidade a teicoplanina) os mais importantes na clínica, visto que são os mais prevalentes e altamente transferíveis para outros patógenos (Dutka-Malen *et al.*, 1995; Perichon *et al.,* 1997; Fines *et al.*, 1999; McKessar *et al.,* 2000; Boyd *et al.,* 2008; Xu *et al.,* 2010; Lebreton *et al.*, 2011).

1.3.2 Epidemiologia do VRE

O primeiro caso de enterococo resistente à vancomicina (VRE) foi descrito em 1986 na Europa (Uttley *et al.,* 1988). A seguir, foram descritos casos nos EUA, e rapidamente se disseminou entre hospitais, pessoas e animais, países e continentes (Hayden, 2000; Bonten *et al.,* 2001; Cattoir & Leclercq, 2013).

No Brasil, o primeiro relato de VRE_{fm} foi em 1996, em um hospital de Curitiba, onde o isolado, além de apresentar resistência à vancomicina, também demonstrou resistência a teicoplanina, ampicilina e altos níveis de resistência para gentamicina e estreptomicina (Dalla Costa *et al*., 1998). Já em 1997, em São Paulo, foi reportado o

primeiro caso de *E. faecium* com fenótipo *vanA* isolado de um paciente com meningite (Zanella *et al.,* 1999).

A partir dos primeiros relatos de VRE, vários casos isolados e surtos foram observados em diferentes estados do Brasil (Zanella *et al.,* 2003; Vilela *et al.,* 2006; d'Azevedo *et al.,* 2008; Gales *et al.,* 2009; Pereira *et al.,* 2010; Palazzo *et al.,* 2011). Na cidade de Porto Alegre, o primeiro relato de VRE foi em 2000, em um paciente internado na UTI de um hospital local (d'Azevedo *et al.,* 2000). Mais recentemente, entre agosto de 2010 e setembro de 2011, foi observado um novo surto em Porto Alegre, onde 29 isolados de VRE_{fm} de diferentes pacientes foram analisados quanto ao seu perfil genômico e todos foram classificados com pertencentes ao mesmo clone (Resende *et al.,* 2014).

Orsi & Ciorba (2013) realizaram uma pesquisa com dados epidemiológicos, e observaram que a epidemiologia de VRE nos EUA e na Europa são aparentemente diferentes. A percentagem de VRE em infecções enterocócicas é menor que 2% na Finlândia e Holanda; 4,2% na Itália; maior que 20% na Irlanda, Grécia e Portugal; e nos EUA chega a 33%.

Até a metade dos anos 80 não havia nenhum caso de VRE_{fm} nos EUA, até o início dos anos 90 o número de isolados não chegava a 1%. Porém, essa realidade foi sendo mudada e a frequência de VRE_{fm} aumentou significativamente, chegando a 80% em 2007 e contrastando com o isolamento *E. faecalis* resistentes a vancomicina que não passou dos 5% (Arias & Murray, 2008; Hidron *et al.,* 2008; Arias & Murray, 2012).

O programa SENTRY realizou um estudo com 12.324 cocos Gram-positivos coletados consecutivamente em hospitais da América Latina, entre janeiro de 2003 a dezembro de 2008. Foi observado que o número de VRE passou de 5% em 2003

para 15,5% em 2008, e que no Brasil o aumento foi mais agravante (de 6,9% a 31,1%) (Sader *et al.,* 2009).

Em 2011, Jones *et al.,* (2013) realizaram um estudo com 4979 diferentes micro-organismos oriundos de 11 países da América Latina (Argentina, Brasil, Chile, Colombia, Costa Rica, Equador, Guatemala, México, Panamá, Peru e Venezuela) com o objetivo de monitorar padrões de susceptibilidade antimicrobiana. A taxa de VRE isolados entre todos os países foi de 14%, sendo que o Brasil apresentou o maior índice de VRE (27%).

A colonização intestinal por VRE parece ser um fator importante para a ocorrência de infecção por esse micro-organismo, embora não seja uma condição *sine qua non*. Lee *et al.* (2013) usaram um modelo matemático para avaliar a disseminação regional e o controle de VRE entre todos os hospitais e comunidades do Condado de Orange (California), e observaram que um aumento de 10% na prevalência da colonização por VRE em qualquer hospital, provoca um aumento médio relativo de 2,8% na prevalência de VRE em todos os outros hospitais, e que hospitais maiores tem um maior efeito sobre outros hospitais. Segundo os autores, isso se deve ao fato de que os pacientes fazem conexões entre hospitais e outros serviços de saúde e não há um monitoramento dessa migração. E sugerem que um programa de vigilância dos pacientes colonizados e dos hospitais compartilhados pode ajudar no controle da disseminação.

1.3.3 *Enterococcus* Não-Susceptíveis à Daptomicina

Daptomicina é um antimicrobiano natural pertencente à classe dos lipopeptídeos cíclicos, derivado da fermentação de *Streptomyces roseosporus,* ativo apenas contra bactérias Gram-positivas. Seu mecanismo de ação envolve ligação

(na presença de íons de cálcio) a membranas bacterianas tanto de células em fase de crescimento como de células em fase estacionária causando despolarização e induzindo a inibição rápida da síntese de proteínas, do DNA e do RNA, resultando na morte da célula bacteriana com lise celular significante.

Esse antimicrobiano tem se tornado um importante agente contra VRE devido a sua potente atividade bactericida *in vitro* (Tran *et al,* 2013), entretanto ainda não foi aprovado pelo *Food and Drug Administration* (FDA) para esta indicação (Arias & Murray, 2012). Casos de enterococos não-susceptíveis a daptomicina têm sido reportados em diferentes partes do mundo, porém ainda pouco se sabe sobre os mecanismos de resistência de enterococos a este antimicrobiano (Mohr *et al.,* 2009; Kelesidis *et al.,* 2011).

Tran e colaboradores (2013) demonstraram que a não susceptibilidade a daptomicina em isolados de *E. faecalis* resistentes à vancomicina está associada a um mecanismo em que a bactéria consegue desviar o antibiótico para longe da região (septo) em que ocorre a divisão celular, permitindo assim a multiplicação bacteriana.

Alguns autores sugerem que a exposição prévia a daptomicina e/ou a cefalosporinas e metronidazóis, são fatores de risco para o desenvolvimento da resistência (Judge *et al.,* 2012; Storm *et al.,* 2012). Outros estudos propõem que, como a daptomicina é utilizada na medicina veterinária em alguns países, animais teriam o desenvolvimento de enterococos não-susceptíveis e estes seriam transferidos ao homem pelo consumo de gêneros alimentíceos contaminados (Zhang *et al.,* 2010; Kelesidis *et al.,* 2012).

O maior concenso observado na literatura relaciona a não susceptibilidade com a metodologia utilizada. De acordo com as normas do *Clinical Laboratory*

Standards Institute (CLSI 2013), é preconizado como padrão-ouro para testar a daptomicina em *Enterococcus* spp. o método de microdiluição em caldo com acréscimo de 50µg/ml de cálcio no meio. Muitos estudos demonstram que se o meio apresentar menos cálcio que o necessário, a concentração minima inibitória pode se elevar. Devido a dificuldade de manter a concentração ideal de cálcio, esse teste apresenta uma melhor acurácia se comparado com outras metodologias comerciais (E-test, Neo-Sensitabs, Microscan), sugerindo a utilização de outros métodos apenas para confirmação dos resultados (Kelesidis *et al.,* 2011; Palavecino *et al.,* 2013).

1.4 Justificativa

O estudo dos biofilmes tem uma grande importância na área clínica, pois está diretamente associado à patogênese de muitas doenças. *Enterococcus faecalis* e *Enterococcus faecium* estão entre as principais causadoras de infecções associadas à assistência à saúde no homem, por isso torna-se relevante o estudo que avalie o mecanismo de formação e funcionamento do biofilme dessas espécies, como forma de compreensão, prevenção e gestão das infecções.

A hipótese é que enterococos obtidos de amostras hospitalares apresentem grande capacidade de formação de biofilmes bem como índices elevados de resistência aos antimicrobianos, o que reflete ainda mais a importância de investigar os mecanismos de virulência desses micro-organismos e relacioná-los com a formação de biofilme e susceptibilidade aos antimicrobianos.

2. REFERÊNCIAS BIBLIOGRÁFICAS

1. Anghinah R, Watanabe RG, Simabukuro MM, Guariglia C, Pinto LF, Gonçalves DC. Native valve endocarditis due to *Enterococcus hirae* presenting as a neurological deficit. Case Rep Neurol Med 2013; 2013:636070.

2. Arias CA, Murray BE. Emergence and management of drug-resistant enterococcal infections. Expert Ver Anti Infect Ther 2008; 6(5): 637-55.

3. Arias CA, Murray BE. The rise of the *Enterococcus:* beyond vancomycin resistance. Nat Rev Microbiol. 2012; 10(4):266-278.

4. Baldassarri L, Cecchini R, Bertuccini L, Ammendolia MG, Iosi F, Arciola CR, *et al*. *Enterococcus* spp. produces slime and survives in rat peritoneal macrophages. Medical Microbiology and Immunology. 2001; 190: 113-20.

5. Bender EA, de Freitas ALP, Barth AL. Avaliação do perfil de suscetibilidade antimicrobiana de *Enterococcus* spp. isolados em dois hospitais de Porto Alegre – RS, Brasil. RBAC 2010; 42(1): 15-19.

6. Bonten MJ, Willems R, Weinstein RA. Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis. 2001; 1:314-25.

7. Boyd DA, Willey BM, Fawcett D, Gillani N, Rulvey MR. Molecular characterization of *Enterococcus faecalis* N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser Gene Cluster, *vanL*. Antimicrob Agents Chemother. 2008; 52(7): 2667-72.

8. Bozdogan B, Leclercq R. Effects of genes encoding resistance to streptogramins A and B on the activity of quinupristin-dalfopristin against *Enterococcus faecium*. Antimicrob Agents Chemother 1999; 43(11): 2720-5.

9. Carabalona JF, Ledochowski S, Rulliat E, Mottard N, Friggeri A. Necrotizing pneumonia due to *Enterococcus faecalis* in an immunocompromised patient of 67 years. Ann Fr Anesth Reanim 2013; 32(2): 887-8.

10. Cattoir V, Leclercq R. Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce? Antimicrob Chemother. 2013; 68: 731–42.

11. Chuang-Smith ON, Wells CL, Henry-stanley MJ, Dunny GM. Acceleration of *Enterococcus faecalis* biofilm formation by aggregation substance expression in an ex vivo model of cardiac valve colonization. Plos One. 2010; 5(12) 1-11.

12. Clinical and Laboratory Standards Institute (CLSI) (2013). Performance standards for antimicrobial susceptibility testing; twenty-third informational supplement M100-S23. Wayne, PA.

13. Costerton JW, Lewandoeski Z, Caldwell DE, Korber DR, Lappin-scott HM. Microbial biofilms. Annu Rev Microbiol. 1995; 49: 711-45.

14. Costerton JW, Lewandoeski Z. The biofilm lifestyle. Advances in Dental Research. 1997; 11(2): 192-5.

15. Creti R, Imperi M, Bertuccini L, Fabretti F, Orefici G, Di Rosa R, *et al.* Survey for virulence determinants among *Enterococcus faecalis* isolated from diferente sources. J Med Microbiol. 2004; 53:13-20.

16. Creti R, Koch S, Fabretti F,f Baldassarri L, Huebner J. Enterococcal colonization of the gastro-intestinal tract: role of biofilm and environmental oligosaccharides. BMC Microbiol. 2006; 6(60): 1-8.

17. Daubié AS, Mayaux J, Epelboin L, de Tymowski C, Mbadi A, Jarlier V, Brossier F. False-positive pneumococcal antigen test in a case of *Enterococcus faecalis* meningitis. Med Mal Infect 2013; 43(11-12): 483-5.

18. d'Azevedo PA, Kacman SB, Achmalfuss T, Silva A, Rodrigues LF. Primeiro caso de *Enterococcus* resistente à vancomicina isolado em Porto Alegre, RS. J Bras Patol. 2000; 36: 258.

19. d'Azevedo PA, Dias CAG, Lemos SK, Bittencourt JAF, Teixeira LM. Antimicrobial Susceptibility among *Enterococcus* isolates from the city of Porto Alegre, RS, Brazil. Braz J Microbiol 2004; 35: 199-204.

20. d'Azevedo PA, Dias CAG, Teixeira LM. Genetic diversity and antimicrobial resistance of enterococcal isolates from southern region of Brazil. Rev Inst Med Trop S Paulo 2006; 48(1): 11-16.

21. d'Azevedo PA, Furtado GH, Medeiros EA, Santiago KA, Silbert S, Pignatari AC. Molecular characterization of vancomycin-resistant enterococci strains eight yeats apart from its first isolation in São Paulo, Brazil. Rev Inst Med Trop São Paulo. 2008; 50(4): 195-8.

22. Dalla Costa LM, Souza DC, Martins LT, Zanella RC, Brandilone MC, Bokermann S, Sader HS, Souza HA. Vancomycin-Resistant *Enterococcus faecium*: First Case in Brazil. Braz J Infect Dis. 1998; 2(3):160-3.

23. Di Rosa R, Creti R, Venditti M, d'Amelio R, Arciola CR, Montanaro L, *et al.* Relationship between biofilm formation, the enterococcal surface protein (*esp*) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. FEMS Microbiol. 2006; 256: 145-50.

24. Donlan RM. Biofilms and device-associated infection. Emerg Infect Dis. 2001; 7(2): 277-81.

25. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002; 15(2): 167-93.

26. Dutka-Malen S, Evers S, Courvalin P. Detection of glicopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995; 33(1): 24-7.

27. Euzéby JP. List of prokaryotic names whit standing in nomenclature – genus *Enterococcus*. Disponível em: http://www.bacterio.cict.fr/e/enterococcus.html. Acesso em 18 de novembro de 2013.

28. Facklan RR, Carvalho MGS, Teixeira LM. History, taxonomy, biochemical characteristics and antibiotic susceptibility testing of enterococci. In: Gilmore MS. (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. Washington DC: Asm Press, 2002; 1-54.

29. Fracalanzza SA, Scheidegeer EM, Santos PF, Leite PC, Teixeira LM. Antimicrobial resistance profiles of enterococci isolated from poultry meat and pasteurized milk in Rio de Janeiro. Mem Inst Oswaldo Cruz. 2007; 102(7): 853-9.

30. Fines M, Perichon B, Reynolds P, Sahm DF, Courvalin P. *vanE*, a new type of acquired glycopeptides resistance in *Enterococcus faecalis* BM4405. Antimicrob Agents Chemother. 1999; 43(9): 2161-4.

31. Fisher K, Phillips C. The ecology, epidemiology and virulence of *Enterococcus*. Microbiol. 2009; 155: 1749-57.

32. Fontana R, Ligozzi M, Pittaluga F, Satta G. Intrinsic penicillin resistance in enterococci. Microb Drug Resist 1996; 2(2): 209-13.

33. Foulquié-Moreno MRF, Sarantinopoulos P, Tsakalidou E, De Vuyst l. The role and application of enterococci in food and health. Int J Food Microbiol. 2006; 106(1): 1-24.

34. Furumura MT, Figueiredo PMS, Carbonell GV, Darini ALC, Yano T. Virulence-associated characteristics of *Enterococcus faecalis* strains isolated from clinical sources. Braz J Microbiol. 2006; 37: 230-6.

35. Gales AC, Sader HS, Ribeiro J, Zoccoli C, Barth A, Pignatari AC. Antimcirobial susceptibility of gram-positive bacteria isolated in Brazilian hospitals participating in the SENTRY Program (2005-2008). Braz J Infect Dis. 2009; 13(2): 90-8.

36. Garsin DA, Willems RJ. Insigths into the biofilm lifestyle of enterococci. Virulence. 2010; 1(4): 219-21.

37. Gilmore MS, Coburn PS, Nallapareddy SR, Murray BE. Enterococcal virulence. In: Gilmore MS. (ed). The enterococci: pathogenesis, molecular

biology, and antibiotic resistance, 1 ed. Washington, DC: American Society For Microbiology, 2002; 301-54.

38. Gilmore MS, Lebreton F, Van Schaik W. Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. Curr Opin Microbiol. 2013; 16: 10-16.

39. Giridhara Upadhyaya PM, Ravikumar KL, Umapathy BL. Review of virulence of *Enterococcus*: an emerging nosocomial pathogen. Indian J Med Microbiol 2009; 27: 301-5.

40. Hayden MK. Insights into the epidemiology and control of infection with vancomycin-resistant enterococci. Clin Infect Dis. 2000; 31:1058–65.

41. Hancock IE, Perego M. Systematic inactivation and phenotipic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583. J Bacteriol. 2004; 186(23): 7951-8.

42. Heikens E, Singh KV, Jacques-Palaz KD, van Luit-Asbroek M, Oostdijk EA, Bonten MJ, *et al.* Contribution of the enterococcal surface protein Esp to pathogenesis of *Enterococcus faecium* endocarditis. Microbes Infect. 2011; 13(14-15): 1185-90.

43. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, *et al*. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare –associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect Control Hosp Epidemiol 2008; 29(11): 996-1011.

44. Iaria C, Stassi G, Costa GB, Di Leo R, Toscano A, Cascio A. Enterococcal meningitis caused by *Enterococcus casseliflavus*. First case report. BMC Infect Dis 2005; 14: 3.

45. Jasovich A, Ganaha MC< Ebi C, García RD, Blanco MA, Lopardo H. Endocarditis due to vancomycin-resistant *Enterococcus raffinosus* successfully treated with linezolid: case report and review of literature. Rev Argent Microbiol 2008; 40(4): 204-7.

46. Jones RN, Guzman-Blanco M, Gales AC, Gallegos B, Castro AL, Martino MD *et al.* Susceptibility rates in Latin American nations: report from a regional resistance surveillance program (2011). Braz J Infect Dis. 2013; 17: 672-681.

47. Jones S, England R, Eyans M, Soo SS, Venjatesan P. Microbiologically confirmed meningoencephalitis due to *Enterococcus avium*: a first report. J Infect 2007; 54: 129-31.

48. Judge T, Pogue JM, Marchaim D, Ho K, Kamatam S, Parveen S, *et al*. Epidemiology of vancomycin-resistant enterococci whit reduced susceptibility to daptomycin. Infect Control Hosp Epidemiol. 2012; 33: 1250-4.

49. Kafil HS, Mobarez AM, Moghadam MF. Adhesion and virulence factors properties of enterococci isolated from clinical samples in iran. Indian J Pathol Microbiol. 2013; 56(3): 238-42.

50. Kanematsu E, Deguchi T, Yasuda M, Kawamura T, Nishino Y, Kawada Y. Alterations in the *gyrA* subunit of DNA gyrase and the *parC* subunit of DNA topoisomerase IV associated whit quinolone resistance in *Enterococcus faecalis.* Antimicrob Agents Chemother 1998; 42(2): 433-5.

51. Kayaoglu G, Orstavik D. Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. Crit Rev Oral Biol Med. 2004; 15(5): 308-20.

52. Kelesidis T, Humphries R, Uslan DZ, Pegues DA. Daptomycin nonsusceptible enterococci: an emerging challenge for clinicians. Clin Infect Dis. 2011; 52: 228-34.

53. Kelesidis T, Humphries R, Uslan DZ, Pegues DA. De novo daptomycin-nonsuscetible enterococcal infections. Emerg Infect Dis. 2012; 18: 674-76.

54. Khan Fy, Elshafi SS. *Enterococcus gallinarum* meningitis: a case report and literature review. J Infect Dev Ctries 2011; 5(3): 231-4.

55. Kurup A, Chlebicki MP, Ling ML, Koh TH, Tan KY, Lee LC, Howe KB. Control of a hospital wide vancomycin-resistant Enterococci outbreak. Am J Infect Control 2008; 36(3): 206-11.

56. Landman D, Quale JM. Management of infections due to resistant enterococci: a review of therapeutic options. J Antimicrob Chemother. 1997; 40(2): 161-70.

57. Leavis H, Top J, Shankar N, Borgen K, Van Embden J, Willems, RJL. A novel putative enterococcal pathogenicity island linked to the *esp* virulence gene of *Enterococcus faecium* and associated whit epidemicity. J Bacteriol. 2004 186(3): 672-82.

58. Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade P, *et al.* D-Ala-D-Ser *vanN*-type transferable vancomycin resistance in *Enterococcus faecium.* Antimicrob Agents Chemother. 2011; 55(10): 4606-12.

59. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, *et al.* Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. MBio. 2013; 4(4): 00534-13.

60. Lee BY, Yilmaz SL, Wong KF, Bartsch SM, Eubank S, Song Y, *et al.* Modeling the regional spread and control of vancomycin-resistant enterococci. Am J Infect Control. 2013; 41(8): 668-73.

61. Lu ZS, Meng L, Liu ZH, Ren GH, Sun AJ, Liu XM. Expression of quorum-sensing related genes during *Enterococcus faecalis* biofilm formation. Zhonghua Kou Qiang Yi Xue Za Zhi. 2013; 48(8): 485-9.

62. Malani PN, Kauffman CA, Zervos MJ. Enterococcal disease, epidemioloy, and treatment. In: Gilmore MS. (ed). The enterococci: pathogenesis, molecular biology, and antibiotic resistance, 1 ed. Washington, DC: American Society for Microbiology, 2002. 385-408.

63. McKessar SJ, Berry AM, Bell JM, Turnidge JD, Paton JC. Genetic characterization of *vanG*, a novel vancomycin resistance locus of *Enterococcus faecalis.* Antimicrob Agents Chemother**.** 2000; 44(11): 3224-28.

64. Mohamed JA, Huang DB. Biofilm formation by enterococci. J Med Microbiol. 2007; 56: 1581-88.

65. Mohr JF, Friedrich LV, Yankelev S, Lamp KC. Daptomycin for the treatment of enterococcal bacteraemia: results from the Cubicin outcomes registry and experience (CORE). Int J Antimicrob Agents. 2009; 33: 543-8.

66. Mondino SS, Castro AC, Mondino PJ, Carvalho MG, Silva KM, Teixeira LM. Phenotypic and genotypic characterization of clinical and intestinal enterococci isolated from inpatients and outpatients in two Brazilian hospitals. Microb Drug Resist 2003; 9:167-74.

67. Murdoch DR, Mirrett S, Harrell LJ, Monahan JS, Reller LB. Sequential emergence of antibiotic resistance in enterococcal bloodstream isolates over 25 years. Antimicrob Agents Chemother. 2002; 46(11): 3676-78.

68. Murray BE. The life and times of the *Enterococcus*. Clin Microbiol Rev. 1990; 3(1): 46-65.

69. Nascimento T, Taveira N. Os biofilmes microbianos como agentes causais de doenças humanas. Biol. 2001; 4:1-6.

70. Orsi GB, Ciorba V. Vancomycin resistant enterococci healthcare associated infections. Ann Ig. 2013; 25(6): 485-92.

71. Palavecino EL, Burnell JM. False daptomycin-nonsusceptible MIC results by Microscan panel 29 relative to Etest results for Staphylococcus aureus and enterococci. J Clin Microbiol. 2013; 51: 281-3.

72. Palazzo IC, Pitondo-Silva A, Levy CE, da Costa Darini AL. Changes in vancomycin-resistant *Enterococcus faecium* causing outbreaks in Brazil. J Hosp Infect. 2011; 79(1): 70-4.

73. Palmer KL, Daniel A, Hardy C, Silverman J, Gilmore MS. Genetic Basis for Daptomycin Resistance in Enterococci. Antimicron Agents Chemother. 2011; 55: 3345-56.

74. Panesso D, Reyes J, Rincón S, Díaz L, Galloway-Peña J, Zurita J, *et al.* Molecular epidemiology of vancomycin-resistant *Enterococcus faecium*: a prospective, multicenter study in south american hospitals. J Clin Microbiol. 2010; 48(5): 1562-9.

75. Patel SN, Memari N, Shahinas D, Toye B, Jamieson FB, Farrel D. Linezolid resistance in *Enterococcus faecium* isolated in Ontario, Canada. Diagn Microbiol Infect Dis 2013; 77(4): 350-3.

76. Pereira GH, Müller PR, Zanella RC, de Jesus castro Lima M, Torchio DS, Levin AS. Outbreak of vancomycin-resistant enterococci in a tertiary hospital: the lack of effect of measures directed mainly by surveillance cultures and differences in response between *Enterococcus faecium* and *Enterococcus faecalis.* Am J Infect Control. 2010; 38(5): 406-409.

77. Perichon B, Reynolds P, Courvalin P. *vanD*-type glicopeptide-resistant *Enterococcus faecium* BM4339. Antimicrob Agents Chemother. 1997; 41(9): 2016-8.

78. Pillai SK, Sakoulas G, Eliopoulos GM, Moellering RC Jr, Murray BE, Inouye RT. Effects of glucose on *fsr*-mediated biofilm formation in *Enterococcus faecalis*. J Infect Dis. 2004; 190(5): 967-70.

79. Prystowsky J, Siddiqui F, Chosay J, Shinabarger DL, Millichap J, Peterson LR, Noskin GA. Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. Antimicrob Agents Chemother.2001; 45(7): 2154-6.

80. Resende M, Caierão J, Prates JG, Narvaez GA, Dias CA, d'Azevedo PA. Emergence of *vanA* vancomycin-resistant *Enterococcus faecium* in a hospital in Porto Alegre, South Brazil. J Infect Dev Ctries. 2014. 8(2): 160-7.

81. Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in combined medical-surgical intensive care units in the United States. Infect Control Hosp Epidemiol. 2000; 21(8): 510-5.

82. Sader HS, Moet GJ, Jones RN. Antimicrobial resistance among Gram-positive bacteria isolated in Latin American hospitals. J Chemother. 2009; 21(6): 611-20.

83. Schleifer KH, Kilpper-Bälz R. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the Genus *Enterococcus* nom. ver. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. Nov. Int J Syst Bacteriol. 1984; 34: 31-34.

84. Sepandj F, Ceri H, Gibb AP, Read RR, Olson M. Biofilm infection in peritoneal dialysis-related peritonitis: comparison of standart mic and mbec in evaluation of antibiotic sensitivy of coagulase-negative staphylococci. Perit Dial Int. 2003; 23(1): 74-94.

85. Shankar V, Baghdayan AS, Huycke MM, Lindahl G, Gilmore MS. Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. Infect Immun. 1999; 67(1): 193-200.

86. Sharifi Y, Hasani A, Ghotaslou R, Varshochi M, Hasani A, Aghazadeh M, Milani M. Survey of virulence determinants among vancomycin resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical specimens of hospitalized patients of north west of Iran. Open Microbiol J. 2012; 6: 34-39.

87. Schlievert PM, Chuang-Smith ON, Peterson ML, Cook LC, Dunny GM. *Enterococcus faecalis* endocarditis severity in rabbits is reduced by IgG Fabs interfering whit aggregation substance. PLos One. 2010; 5(10): e13194.

88. Shepard BD, Gilmore MS. Antibiotic-resistant enterococci: the mechanisms and dynamics of drug introduction and resistance. Microbes Infect. 2002; 4(2): 215-24.

89. Shirano M, Takakura S, Yamamoto M, Matsumura Y, Matsushima A, Nagao M, *et al.* Regional spread of vanA- or vanB- positive *Enterococcus gallinnarum* in hospitals and long-term care facilities in Kyoto prefecture, Japan. Epidemiol Infect 2011; 139(3): 430-6.

90. Shorman M, Al-Tawfiq JA. Risk factors associated whit vancomycin-resistant *Enterococcus* in intensive care unit settings in Saudi Arabia. Interdiscip Perspect Infect Dis. 2013; 2013:369674.

91. Soltani M, Beighton D, Philpott-Howard J, Woodford N. Mechanisms of resistance to quinupristin-dalfopristin among isolates of *Enterococcus faecium* from animals, raw meat, and hospital patients in Western Europe. Antimicrob Agents Chemother 2000; 44(2): 433-6.

92. Sydnor ER, Perl TM. Hospital epidemiology and infection control in acute-care settings. Clin Microbiol Rev. 2011; 24(1): 141-73.

93. Storm JC, Diekema DJ, Kroeger JS, Johnson SJ, Johannsson B. Daptomycin exposure precedes infection and/or colonization whit daptomycin nonsusceptible enterococcus. Antimicrob Resist Infect Control. 2012; 1: 1-7.

94. Tavares W. Problems with Gram-positive bacteria: resistance in staphylococci, enterococci, and pneumococci to antimicrobial drugs. Rev. Soc. Bras. Med. Trop. 2000; 33(3): 281-301.

95. Teixeira LM, Carvalho MGS, Shewmaker PL, Facklam RR. Enterococcus. In: Versalovic J, Carrol KC, Funke G, Jorgensen JH, Landry ML, Warnock DW. Manual of Clinical Microbiology, 10th ed., v. 1, American Society for Microbiology, Washington, DC. 2011, 350-364.

96. Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar M. Enterococcal surface protein, *Esp*, enhances biofilm formation by *Enterococus faecalis.* Infec. Immun. 2004; 72: 6032-9.

97. Titze-de-Almeida R, Rollo Filho M, Silveira CAN, Rodrigues IP, Eudes Filho J, Nascimento RS, *et al.* Molecular epidemiology and antimicrobial susceptibility from Brazilian Intensive Care Units. Braz J Infect Dis 2004; 8(3): 197-205.

98. Tran TT, Panesso D, Mishra NN, Mileykovskaya E, Guan Z, Munita JM, *et al*. Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. MBio. 2013; 23: 1-10.

99. Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, *et al*. The enterococcal surfasse protein, *esp*, is involved in *Enterococcus faecalis* biofilm formation. Appl Environ Microbiol. 2001; 67(10): 4538-45.

100. Umgelter A, Prinz C, Gaa J, Huber W. Ascending pneumonia complicating endoscopic therapy of a pancreativ abscess. Endoscopy 2007; 39: 267.

101. Uttley AH, Collins CH, Naidoo J, George RC. Vancomycin-resistant enterococci. Lancet. 1988; 1(8575-6): 57-8.

102. Vilela MA, Souza SL, Palazzo IC, Ferreira JC, Morais MA Jr, Darini AL, Morais MM. Identification and molecular characterization of van A-type vancomycin-resistant *Enterococcus faecalis* in Northeast of Brazil. Mem Inst Oswaldo Cruz. 2006; 101(7): 715-9.

103. Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, *et al. vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium.* Antimicrob Agents Chemother. 2010; 54(11): 4643-7.

104. Zanella RC, Valdetaro F, Lovgren M, Tyrrel GJ, Bokermann S, Almeida SC, Vieira VS, Brandileone MC. First confirmed case of a vancomycin-resistant *Enterococcus faecium* whit vanA phenotype from Brazil: isolation from a meningitis case in São Paulo. Microb Drug Resist. 1999; 5(2): 159-62.

105. Zanella RC, Brandileone MC, Bokermann S, Almeida SC, Valdetaro F, Vitório F, *et al*. Phenotypic and genotypic characterization of vanA *Enterococcus* isolated during the first nosocomial outbreak in Brazil. Microb Drug Resist. 2003; 9(3): 283-91.

106. Zhang J, Wall SK, Xu L, Ebner PD. Contamination Rates and Antimicrobial Resistance in Bacteria Isolated from “Grass-Fed” Labeled Beef Products. Foodborne Pathog Dis. 2010 7: 1331-6.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar as características fenotípicas e genotípicas de isolados de *Enterococcus faecalis* e *Enterococcus faecium* obtidos a partir de amostras clínicas, de pacientes internados no CHSCMPA (Complexo Hospitalar Santa Casa de Misericórdia de Porto Alegre).

3.2 Objetivos específicos

1) Caracterizar a distribuição das espécies de enterococos isoladas a partir de diferentes amostras clínicas obtidas de pacientes internados no CHSCMPA no período estudado (setembro de 2012 a agosto de 2013).
2) Avaliar o perfil de susceptibilidade dos isolados aos antimicrobianos comumente utilizados no tratamento, através dos métodos de detecção da concentração inibitória mínima (CIM) e verificar a relação clonal entre os isolados de *Enterococcus* que apresentarem resistência a vancomicina.
3) Verificar a produção de biofilme entre os diferentes sítios de onde os isolados foram obtidos.
4) Avaliar os fatores de virulência através da presença de genes *esp* e esp_{efm}, *gelE* e *agg* e associar com a formação do biofilme.

4. DESENVOLVIMENTO

A seção de desenvolvimento será apresentada a seguir na forma de dois manuscritos. O primeiro manuscrito foi submetido à publicação na revista *Virulence* e é intitulado **Correlation of biofilm formation and presence of *gelE*, *esp* and *agg* in clinical isolates of *Enterococcus* spp.** O segundo manuscrito será submetido à publicação no periódico *Memórias do Instituto Osvaldo Cruz* e é intitulado **Clonal Spread of Vancomycin resistant *Enterococcus faecium* Daptomycin-nonsusceptible isolated in Southern Brazil.**

4.1 Manuscrito I

Correlation of biofilm formation and presence of *gelE*, *esp* and *agg* in clinical isolates of *Enterococcus* spp.

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Abstract

Enterococci have been reported as a major cause of nosocomial infections. Biofilm formation and diverse virulence factors have been described as contributing to the enterococci fitness and its persistence as pathogen. Based on that, this study aimed to evaluate the ability of *Enterococcus* spp to form biofilm, and to observe its correlation with antimicrobial resistance and virulence. Two hundred and forty *Enterococcus* strains were obtained: 92.1% *Enterococcus faecalis* and 7.9% *Enterococcus faecium*. Multidrug resistance was observed in 10% of isolates (66.6% *E. faecium* and 33.3% *E. faecalis*). Ability to form biofilm was observed in 81.25% of isolates, but *E. faecium* were mostly non-adherent than *E. faecalis*. The virulence genes *gelE, esp* and *agg* were found in 73.3%, 70% and 58.3% of the isolates, respectively, and there was a significant correlation among each virulence gene and biofilm formation ($p<0.05$). In this study, *E. faecalis* harbored more virulence factors and *E. faecium* showed higher antibiotic resistance, confirming that each species demonstrate distinct potential for initiating an infection.

Keyword: biofilm, *esp, agg, gel*E, *Enterococcus faecalis, Enterococcus faecium*

Introduction

Enterococci have been reported as one of the leading cause of nosocomial infections. *Enterococcus faecalis* is, by far, the species most frequently isolated, commonly recovered from surgical wound, intra-abdominal, bloodstream and mainly, urinary tract infections (UTIs).[1,2]

Some virulence factors have been described as contributing to the enterococci fitness and its persistence as pathogen in the infection site. These factors are involved mainly with ability to form biofilm, as gelatinase (encoded by *gelE*), enterococcal surface protein (encoded by *esp*) and aggregation substance (encoded by *agg)*.[3,4]

Besides their involvement with biofilm formation, GelE is also capable of hydrolyzing collagen, casein and hemoglobin[5,6,7] and Agg mediates formation of aggregates during conjugation[8], promoting the transference of mobile genetic elements and contributing to pathogenesis.[9,10,11]

Biofilm is a structured and complex community of microorganisms adhered to a biotic or abiotic surface.[12] The ability of forming biofilm is a vital strategy of all kinds of microrganisms, which allow them surviving under adverse situations. Enterococci have an extraordinary ability to form biofilms and this characteristic has been strongly related with specific health conditions, like urinary tract infections.[11,13]

In this context, the aim of this study was to evaluate the ability of *Enterococcus* spp. to form biofilm and observe its possible association with the presence of virulence factors and antimicrobial resistance.

Results

During the study period, 240 *Enterococcus* spp. were recovered: 92.1% (n=221) *Enterococcus faecalis* and 7.9% (n=19) *Enterococcus faecium*. The different sites of isolation are described in Table 1. *Enterococcus faecalis* were particularly recovered from noninvasive sites (89.1%; n=197), while *E. faecium* were most commonly associated with invasive sites (63.1%; n=12). One hundred and sixty-five isolates (68.75%) were from women and the average age of all patients was 52 years-old (from 0 to 98 years old). One hundred and one patients (42.08%) presented more than 61 years.

Table 2 presents susceptibility patterns based on MIC and disk diffusion methods. Among *E. faecalis*, higher rates of resistance were observed to fluoroquinolones and aminoglycosides (high level). *E. faecium* presented higher resistance rates to all antimicrobial classes, with 63.2% of vancomycin resistance. The rate of multiresistance was 10% (n=24), of which, 66.6% (n=16) were *E. faecium* and 33.3% (n=8) *E. faecalis*.

The distribution of virulence factors among isolates is shown in Table 3. Overall, 73.3% harbored gelE, 70% harbored esp, and 58.3% harbored agg. Only 42.6% of the isolates positive to gelE produced gelatinase. Although no statistically significant (p=0.09), esp was more frequent among isolates of noninvasive sites for both species. Isolates from non invasive sites were biofilm formers more frequently than the ones recovered from invasive sites ($p<0.0001$). On the other hand, the other virulence genes and production of gelatinase were randomly distributed in invasive and noninvasive sites (no statistical significance). Among *E. faecium*, *gelE* and gelatinase, even in a very low number, were only found in isolates recovered from invasive sites and *agg* was not found in any strain.

The ability to form biofilm was observed in 81.25% (n=195) of all isolates: 75.4% (n=147) were classified as moderately or strongly adherent and 24.6% (n=48) as weakly

adherent. Only 2 (10.5%) *E. faecium* showed ability to form biofilm, and they were weakly adherent.

Association between each virulence gene and biofilm formation is demonstrated in Table 3. All associations (*gelE*+/biofilm+, *esp*+/biofilm+ and *agg*+/biofilm+) were statistically significant ($p=0.009$, $p<0.001$ and $p=0.001$, respectively). Six isolates (2.5%) presented all virulence genes and did not form biofilm and 4 isolates (1.67%) were biofilm producers and no virulence genes were detected.

Among isolates able of forming biofilm, the highest resistance rates were 22.05% and 17.44% to high level resistance to gentamicin and ciprofloxacin, respectively. For other antimicrobials (linezolid, ampicillin, vancomycin and nitrofurantoin), few biofilm formers were resistant (6.66%; 2.56%; 1.03% and 0.51%, respectively). There were no statistically significant differences between adherent/non-adherent isolates and antimicrobial resistance ($p=0.29$).

Discussion

This study evaluated the ability of forming biofilm, the presence of some virulence genes and the antimicrobial resistance profile in *Enterococcus* spp. In accordance with previous studies in Porto Alegre, Brazil and around the world, characteristics of the target microorganism were corroborated: more recovery from urinary site, overwhelming prevalence of *E. faecalis* (and also regarding biofilm formation) and the peculiarly higher antimicrobial resistance of *E. faecium* comparing to *E. faecalis*.[22-32]

Most of virulence genes searched in our study are related with biofilm formation, specifically in the adhesion step. One could suppose that for invasive isolates, other virulence traits may be more relevant than adherence. For example, the capacity of degrade collagen

and other proteins may be extremely relevant in the invasion and dissemination process. It may justify the association we found among invasive isolates and gelE gene and its product.

We observed that *E. faecalis* produced biofilm more often than *E. faecium*, as previously reported.[13,33] Although not statistically significant (p=0.29), biofilm producers showed lower resistance rates compared with non-adherent isolates, probably due to bacterial fitness. It is important to point out that cells were exclusively tested in their planktonic form. The MICs of cells in biofilm were not determined.

In our study, *esp* was the only virulence gene present in *E. faecium*. Esp is also an important feature in a special lineage of *E. faecium* designated clonal-complex 17 (CC17) that is strongly associated with hospital outbreaks around the world.[32,36] Many authors suggest this is an important molecule for biofilm formation.[7] For example, Di Rosa[33] suggests that there is a synergism between *esp* and biofilm formation, as an advantage to the establishment of successful infection. However, it seems not to be essential for the occurrence of biofilm[36], which have been demonstrating to be a multifactorial process.

Our study presents limitations, as the small number of *E. faecium* isolates. Even though, this study reinforces the well-known characteristics of *E. faecalis* and *E. faecium* as being species with high virulence and resistance, respectively. It also corroborate to the idea of biofilm as a multifactorial process, with the participation of different genes and their products. Studies like this are essential to determinate the role of each molecules in the virulence process and to understand local epidemiological characteristics of enterecocci.

Material and methods

Bacterial strains

Enterococci were consecutively recovered from infections or surveillance sites from patients attended at Complexo Hospitalar Santa Casa de Porto Alegre (Porto Alegre, Brazil) as part of an epidemiological surveillance study (September 2012 to March 2013). Genus identification was performed through the observation of phenotypic characteristics according to Teixeira et al. (2011).[14] Species identification was determined by duplex PCR assay as previously described[15], containing: 5 pmol of the *E. faecalis*-specific primers and 1.25 pmol of the *E. faecium*-specific primers, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) and 0.625 U of Taq DNA polymerase. Amplifications were carried out in a thermocycler Life Pro (Bioer) using 54°C as the annealing temperature.bThe presence of virulence genes (*esp, gelE*, and *agg*) were investigated by PCR, as previously described.[16,17] As a positive control strain was used *Enterococcus faecalis* ATCC 29212 and *Enterococcus faecium* SS1274. Production of gelatinase was phenotypically determined according to Marra et al.[18], using BHI (Brain Heart Infusion) with 4% gelatin.

Susceptibility testing

Susceptibility tests were performed according to CLSI (2013)[19]. Ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (120 µg), linezolid (30 µg) and quinupristin-dalfopristin (15 µg) were tested by disk diffusion (nitrofurantoin 300 µg was tested for urinary site). Minimum Inhibitory Concentration (MIC) by broth microdilution method (BMD) was determined for vancomycin.[19] Multidrug-resistant (MDR) isolates were defined as the ones presenting resistance to three or more different antimicrobial classes.[20]

Biofilm formation

Biofilm formation was performed on polystyrene microtiter plates and optical density results were interpreted as described previously.[21] First, 180 µL of trypticase soya broth

(Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with glucose 1.5% was added to each well of a sterile 96-well polystyrene microtiter plate, followed by 20 μL of bacterial suspension. The plates were incubated for 24 h at 35 ± 2 °C under static conditions. After incubation and broth removal, wells were washed 3 times with sterile saline and bacteria attached were fixed with methanol for 20 min. Adherent bacteria were stained with crystal violet 0.5% for 15 min and biofilm was eluted with ethanol for 30 min. Absorbance was measured at 492 nm using the microtiter plate reader Expert Plus (ASYS Hitech, Eugendorf, Austria). As a positive control strain was used *Staphylococcus epidermidis* ATCC 35984.

Statistical analysis

All analysis were performed using SPSS version 19.0 (USA). Chi-squared test (or Fisher's exact test, when appropriate) was performed and $p<0.05$ were considered statistically significant.

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References

1. Malani PN, Kauffman CA, Zervos MJ. Enterococcal disease, epidemioloy, and treatment. In: Gilmore MS. (ed). The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance, 1 ed. Washington, DC: American Society for Microbiology, 2002. 385-408.

2. Giridhara Upadhyaya PM, Umapathy BL, Ravikumar KL. Comparative study for the presence of enterococcal virulence factors gelatinase, hemolysin and biofilm among clinical and commensal isolates of *Enterococcus faecalis*. J Lab Physicians 2010; 2:100-4.
3. Kafil HS, Mobarez AM, Moghadam MF. Adhesion and virulence factor properties of Enterococci isolated from clinical samples in Iran. Indian J Pathol Microbiol 2013; 56:238-42.
4. Heikens E, Bonten MJ, Willems RJ. Enterococcal surface protein Esp is important for biofilm of *Enterococcus faecium* E1162. J Bacteriol 2007; 189:8233-40.
5. Hancock LE, & Perego M. The *Enterococcus faecallis fsr* two-component system controls biofilm development through production of gelatinase. J Bacteriol 2004; 186:5629-39.
6. Giridhara Upadhyaya PM, Ravikumar KL, Umapathy BL. Review of virulence of enterococcus: an emerging nosocomial pathogen. Indian J Med Microbiol 2009; 27:301-5.
7. Shankar V, Baghdayan AS, Huycke MM, Lindahl G, Gilmore MS. Infection-derived *Enterococcus faecalis* strains are enriched in esp, a gene encoding a novel surface protein. Infection and Immunity 1999; 67:193-200.
8. Chuang-Smith ON, Wells CL, Henry-Stanley MJ, Dunny GM. Acceleration of *Enterococcus faecium* biofilm formation by aggregation substance expression in an ex vivo model of cardiac valve colonization. PLos One 2010; 5:e15798.
9. Gilmore MS, Coburn PS, Nallapareddy SR, Murray BE. Enterococcal Virulence. In: Gilmore MS. (ed). The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance, 1 ed. Washington, DC: American Society for Microbiology, 2002. 301-54.

10. Koch S, Hufnagel M, Theilacker C, Huebner J. Enterococcal infections: host response, therapeutic, and prophylactic possibilities. Vaccine 2004; 22:822-30.
11. Fisher K & Phillips C. The ecology, epidemiology and virulence of *Enterococcus*. Microbiology 2009; 155:1749-57.
12. Tenke P, Köves B, Nagy K, Hultgren SJ, Mendling W, Wullt B et al. Update on biofilm infections in the urinary tract. World J Urol 2012; 30:51-7.
13. Mohamed JA & Huang DB. Biofilm formation by enterococci. Journal of Medical Microbiology 2007; 56:1581-88.
14. Teixeira LM, Carvalho MGS, Shewmaker PL, Facklam RR. *Enterococcus*. In: Versalovic, J.; Carrol, K.C.; Funke, G.; Jorgensen, J.H.; Landry, M.L.; Warnock, D.W. Manual of Clinical Microbiology, 10th ed., v. 1, American Society for Microbiology, Washington, DC. 2011, 350-364.
15. Kariyama R, Mitsuhata R, Chow JW, Clewel DB, Kumon H. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J Clin Microbiol 2000; 38:3092-95.
16. Eaton TJ & Gasson MJ. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microbiol 2001; 67:1628-35.
17. Creti R, Imperi M, Bertuccini L, Fabretti F, Orefici G, Di Rosa R, Baldassarri L. Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. Med Microbiol 2004; 53:13-20.
18. Marra A, Dib-Hajj F, Lamb L, Kaczmarek F, Shang W, Beckius G, et al. Enterococcal virulence determinants may be involved in resistance to clinical therapy. Diagnostic Microbiology and Infectious Disease 2007; 58:59-65.

19. Clinical and Laboratory Standards Institute (CLSI) (2013). Performance standards for antimicrobial susceptibility testing; twenty-third informational supplement M100-S23. Wayne, PA.

20. Magiorakos AP, Srinivasan A, Carey RB, Falagas ME, Giske CG, Harbarth S et al. Multidrug-resistant, extensively, drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012; 18:268-81.

21. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 2000; 40:175-79.

22. Mondino SS, Castro AC, Mondino PJ, Carvalho MG, Silva KM, Teixeira LM. Phenotypic and genotypic characterization of clinical and intestinal enterococci isolated from inpatients and outpatients in two Brazilian hospitals. Microb Drug Resist 2003; 9:167-74.

23. d'Azevedo PA, Dias CA, Teixeira LM. Genetic diversity and antimicrobial resistance of enterococcal isolates from Southern region of Brazil. Rev Inst Med Trop São Paulo 2006; 48:11-6.

24. Titze-de-Almeida R, Rollo Filho M, Nogueira CA, Rodrigues IP, Eudes Filho J, Nascimento RS et al. Molecular epidemiology and antimicrobial susceptibility of Enterococci recovered from Brazilian intensive care units. Braz J Infect Dis 2004; 8:197-205.

25. Bender EA, de Freitas AL, Reiter KC, Lutz L, Barth AL. Identification antimicrobial resistance and genotypic characterization of *Enterococcus* spp. isolated in Porto Alegre, Brazil. Braz J Microbiol 2009; 40:693-700.

26. Conceição N, Oliveira CC, Silva PR, Avila BG, Oliveira AG. Trends in antimicrobial resistance among clinical isolates of enterococci in a Brazilian tertiary hospital: a 4-year study. Rev Soc Bras Med Trop 2011; 44:177-181.

27. Parameswarappa J, Basavaraj VP, Basavaraj CM. Isolation, identification, and antibiogram of enterocci isolated from patients whit urinary tract infection. Ann Afr Med 2013; 12:176-81.

28. Sharifi Y, Hasani A, Ghotaslou R, Naghili B, Aghazadeh M, Milani M, Bazmany A. Virulence and antimicrobial resistance in enterococci isolated from urinary tract infections. Adv Pharm Bull 2013; 3(1): 197-201.

29. Fernandes SC & Dhanashree B. Drug resistance and virulence determinants in clinical isolates of *Enterococcus* species. Indian J Med Res 2013; 137:981-85.

30. Swaminathan S & Alangaden GJ. Treatment of resistant enterococcal urinary tract infections. Curr Infect Dis Rep 2010; 12:455-64.

31. Lee G. Ciprofloxacin resistance in *Enterococcus faecalis* strains isolated from male patients whit complicated urinary tract infection. Korean J Urol 2013; 54:388-93.

32. Willems RJ, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJM. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. Emerg Infect Dis 2005; 11: 821-8.

33. Di Rosa R, Creti R, Venditti M, D'Amelio R, Arciola CR, Montanaro L, Baldassarri L. Relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. FEMS Microbiol Lett 2006; 256:145-150.

34. Hällgren A, Claesson C, Saeedi B, Monstein HJ, Hanberger H, Nilsson LE. Molecular detection of aggregation substance, enterococcal surface protein, and cytolysin genes

and in vitro adhesion to urinary catheters of *Enterococcus faecalis* and *E. faecium* of clinical origin. Int J Med Microbiol 2009: 299:323-332.

35. Comerlato CB, de Resende MC, Caierão J, d'Azevedo PA. Presence of virulence factors in *Enterococcus faecalis* and *Enteroccus faecium* susceptible and resistant to vancomycin. Mem Inst Oswaldo Cruz 2013; 108:590-5.
36. Panesso D, Reyes J, Rincón S, Díaz L, Galloway-Peña J, Zurita J et al. Molecular epidemiology of vancomycin-resistant *Enterococcus faecium*: a prospective, multicenter study in South American hospitals. J Clin Microbiol 2010; 48: 1562-9.

Table 1. Different sites from isolation of *Enterococcus faecalis* and *Enterococcus faecium*

Sites	*E.faecalis* n (%)	*E.faecium* n (%)	Total n (%)
Urine	197 (82.08)	6 (2.5)	203 (84.58)
Blood	10 (4.17)	7 (2.91)	17 (7.08)
Abdominal Secretion	7 (2.91)	1 (0.42)	8 (3.33)
Catheter	1 (0.42)	1 (0.42)	2 (0.84)
Pelvic collection	2 (0.83)	0 (0)	2 (0.84)
Others *	4 (1.67)	4 (1.67)	8 (3.33)
Total	221 (92.1)	19 (7.9)	240 (100)

* sites with one isolate each (bile, bone, secretion perifratura, perihepática puncture, cervical fluid, pleural fluid, rectal fluid, sputum)

Table 2. Susceptibility profile of enterococcal isolates, according to the species

Antibiotics	*E. faecalis* n (%)			*E. faecium* n (%)			Total n (%)		
	S	I	R	S	I	R	S	I	R
Ampicillin	218 (98.6)	-	3 (1.4)	2 (10.5)	-	17 (89.5)	220 (91.7)	-	20 (8.3)
Gentamicin	176 (79.6)	-	45 (20.4)	15 (78.9)	-	4 (21.1)	191 (79.6)	-	49 (20.4)
Ciprofloxacin	118 (53.4)	67 (30.3)	36 (16.3)	-	1 (5.3)	18 (94.7)	118 (49.2)	68 (28.3)	54 (22.5)
Nitrofurantoin	196 (99.5)	1 (0.5)	-	1 (16.7)	1 (16.7)	4 (66.7)	197 (96.6)	2 (0.9)	5 (2.5)
Vancomycin	213 (96.4)	8 (3.6)	-	7 (36.8)	-	12 (63.2)	220 (91.7)	8 (3.3)	12 (5)
Linezolid	204 (92.3)	4 (1.8)	13 (5.9)	19 (100)	-	-	223 (92.9)	4 (1.7)	13 (5.4)
Quinupristin/ Dalfopristin	NT	NT	NT	15 (79)	2 (10.5)	2 (10.5)	15 (79)	2 (10.5)	2 (10.5)

S= Sensitive, I= Intermediate, R= Resistant, NT= not tested

Table 3. Presence of virulence factors in isolates of *E. faecalis* and *E. faecium* recovered from different anatomical sites

Species	Site	n (%)	*gelE*	gelatinase	*esp*	*agg*	Biofilm*
E. faecalis	Invasive	24 (10)	20 (83.3)	8 (33.3)	14 (58.3)	16 (66.6)	20 (83.3)
	Noninvasive	197 (82.1)	154 (78.2)	66 (33.5)	141 (71.6)	124 (62.9)	173 (87.8)
E. faecium	Invasive	12 (5)	2 (16.7)	1 (8.3)	7 (5.8)	-	1 (8.3)
	Noninvasive	7 (2.9)	-	-	6 (85.7)	-	1 (14.3)
Total		240 (100)	176 (73.3)	75 (31.25)	168 (70)	140 (58.33)	195 (81.25)

* Association between biofilm formation and invasive / noninvasive sites ($p<0.0001$)

Table 4. Association between the presence of virulence factors and biofilm formation by Chi-squared test

Virulence Factors	Biofilm n (%)		p value
	Positive	Negative	
gelE +	150 (62.5)	26 (10.8)	0.009
gel E -	45 (18.8)	19 (7.9)	
esp +	147 (61.25)	21 (8.75)	<0.001
esp -	48 (20)	24 (10)	
agg +	124 (51.7)	16 (6.6)	0.001
agg -	71 (29.6)	29 (12.1)	

4.2 Manuscrito II

Clonal Spread of Vancomycin resistant *Enterococcus faecium* Daptomycin-nonsusceptible isolated in Southern Brazil

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Abstract

VRE_{fm} has become increasingly common and their clonal spread has been described in different regions of the world. This work determined the susceptibility profile to antimicrobial agents and the clonal relationships by PFGE of isolates recovered from infections or surveillance sites from inpatients attended at general Hospital in Porto Alegre, Brazil. Four hundred forty-one enterococci were consecutively recovered and among these, 27 were characterized as VRE_{fm}. All twenty seven isolates exhibited resistance to vancomycin and teicoplanin (vanA), ampicillin, ciprofloxacin, and susceptibility to linezolid and quinupristin-dalfopristin. HLR-Ge was detected in 14.8% of the isolates. Five isolates were daptomycin-nonsusceptible (DNSE) by MIC. The PFGE profile analysis enabled us to characterize a main clone referred to as clonal complex A, which grouped 24 isolates including the 5 isolates daptomycin-nonsusceptible. By comparing the clonal relationship of these isolates with an isolate VRE_{fm} of 2011, we observe a very close clonal relationship, suggesting that this strain may be spreading in the city's hospitals. We firstly reported cases of DNSE in Brazil, demonstrating the importance of systematic epidemiological studies to accompany and prevent dissemination of some specific and worrisome phenotypes/genotypes between health institutions of the city.

Keyword: vancomycin-resistant *Enterococcus faecium*, daptomycin-nonsusceptible, PFGE, clonal spread.

Introduction

It is well-established the remarkable ability of *Enterococcus faecium* to rapidly acquire mobile genetic elements associated to antimicrobial resistance. (Willems et al. 2009, Palmer et al. 2011; Lebreton et al. 2013). Vancomycin-resistant *Enterococcus faecium* (VRE_{fm}) has become increasingly common and their clonal spread has been described in different regions of the world (Willems et al. 2005; Cattoir & Leclercq 2013), including Brazil (d'Azevedo et al. 2008; Palazzo et al. 2011, da Silva et al. 2012)

Among genes encoding glycopeptides resistance, the most important is *vanA* since it is the most prevalent around the world and it is highly transferable to other pathogens (Dutka-Malen et al. 1995). VanA-related VRE frequently present multirresistance patterns which considerably reduces therapeutical choices, leading to difficulties in the treatment of infections. In many of these cases, the available effective drugs may be restricted to linezolid, quinupristin-dalfopristin and daptomycin.

The lipopeptide daptomycin had become an alternative therapy for vancomycin-resistant enterococcal infections (Tran et al. 2013). However, It have been reported cases of daptomycin-nonsusceptible enterococci (DNSE), and little is known about the mechanisms of resistance of enterococci to this antimicrobial (Mohr et al. 2009; Kelesidis et al. 2011; Kelesidis et al. 2012).

The objective of this work was to determine the susceptibility profile of VRE_{fm} recovered from inpatients in Porto Alegre, Southern Brazil and the genetic relatedness of these isolates was also determined.

Material and methods

Bacterial strains

From September 2012 to August 2013, consecutive *Enterococci* were recovered from infections or surveillance sites of inpatients attended at Complexo Hospitalar Santa Casa de Porto Alegre (Porto Alegre, Brazil) as part of an epidemiological surveillance study. Among these isolates, the ones presenting resistance to vancomycin were selected for the present study. Besides, for comparison of clonal diversity, it was included an isolate (VRE_{fm}/11) belonging to an outbreak previously characterized in Porto Alegre (2011) in another health institution (Resende et al. 2014).

Genus identification was performed through the observation of phenotypic characteristics according to Teixeira and co-workers (2011). The species identification and the presence of genes *vanA* and *vanB* were determined by PCR as previously described (Kariyama et al. 2000). The four primers sets,shown in Table 1, were added to two different duplex PCR assay: the first containing 5 pmol of *vanA* primers and 2.5 pmol of *vanB;* the second reaction containing 5 pmol of the *E. faecalis*-specific primers and 1.25 pmol of the *E. faecium*-specific primers. The PCR assay was performed in a total volume of 25 µl containing 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) and 0.625 U of Taq DNA polymerase. Amplifications were carried out in a thermocycler Life Pro (Bioer) using 54°C as the annealing temperature. As a positive control strain was used *Enterococcus faecalis* ATCC 29212 and *Enterococcus faecium* SS1274.

Susceptibility testing

Susceptibility tests were performed according to CLSI (2013). Disk diffusion was done for the following antimicrobials: ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (120 µg), linezolid (30 µg), nitrofurantoin (300 µg), quinupristin-dalfopristin (15 µg) and teicoplanin (30 µg). Minimum Inhibitory Concentrations (MIC) was determined by broth microdilution method (BMD) for daptomycin, linezolid and vancomycin. Mueller-hinton cation-adjusted was used for BMD, as recommended by CLSI (2013) guidelines, and the concentration of calcium into the broth was dosed using ion-selective electrodes (Cobas® B 121 system - Roche OMNI ® C). Prediffusion, where antimicrobial has a period of 24 hours of pre-diffusion onto agar before inoculum are plated, was performed according to manufacturer's instruction. For this purpose, tablets containing 30 µg of daptomycin and 100 µg of calcium (Neo-Sensitabs™ - Rosco Diagnostica, Taastrup, Denmark) were placed into the Mueller-Hinton agar, maintained for 2 hours at room temperature and removed. After, plate was maintained for 22 more hours at room temperature, and then, a 0.5 McFarland inoculum was plated into the agar and incubated at 37°C overnight. Isolates with diameter zones lower than 12 mm were considered resistant. Multidrug resistance (MDR) strains were defined as the ones presenting resistance to three or more different antimicrobial classes (Magiorakos et al. 2012).

Chromosomal analysis of genomic DNA by PFGE

The pulsed-field gel electrophoresis (PFGE) was performed as previously described (Teixeira et al. 1997; Mondino et al. 2003) and interpreted according to criteria of Tenover and co-workers *(*1995). The agarose blocks were prepared and treated whit 1 mg/mL of lysozyme (Sigma Co., 48.000U/mg), 5U/mL of mutanolysin

(Sigma Co., 2.000U/mL) and 0.1mg/mL of proteinase K (Sigma Co., 10-20U/mg). Digestion of chromosomal DNA was reached whit 20 U of *SmaI* (New England BioLabs).

Analyses were performed using the automated system Quantity One 1-D Analysis Software (Bio-Rad). Similarity matrices and dendrograms, constructed on SPSS version 19.0 (USA), were obtained by the unweighted pair group method using arithmetic averages (UPGMA). Dice coefficient was used to determine similarity. Isolates presenting 80% or higher similarity were considered as a clone.

Results

During the study period, 441 *Enterococci* were consecutively recovered. Among these isolates, 6.1% (n=27) were characterized as VRE_{fm}. The most common VRE_{fm} related site (table 2) was blood (12 isolates; 44.4%), followed by urine (9 isolates; 33.3%), abdominal secretions (2 isolates; 7.4%) and catheter (1 isolate; 3.7%). One isolate (3.7%) was recovered from pleural fluid and 2 isolates (7.4%) were associated with surveillance culture (rectal swabs). Sixteen isolates (59.3%) were from women and the average age of all patients was 55 years-old. Thirteen isolates (48.1%) were from patients hospitalized in the Intensive Care Unit (ICU) and 69% (n=9) of those presented bloodstream infections.

Antimicrobial susceptibility profile of isolates is summarized in table 2. In our study, all VRE_{fm} were resistant to at least three different antimicrobial classes. All twenty-seven VRE_{fm} exhibited vancomycin MICs higher than 256 μg/mL and resistance to teicoplanin (all carrying *vanA* gene). Also, all isolates were resistant to ampicillin, ciprofloxacin, and susceptible to linezolid (MIC ≤ 4) and quinupristin-dalfopristin. High-Level Resistance to Gentamycin was detected in four (14.8%)

isolates. Five VRE_{fm} (18.5%) were daptomycin-nonsusceptible, presenting MIC ≥ 8 µg/mL. The Neo-sensitabs prediffusion to daptomycin was performed for 25 isolates. Although it confirmed resistance detected by MIC (n=5), it presented false resistance to 19 VRE_{fm} (79.1%), which had MIC ≤ 4. Among DNSE, the majority (60%; n= 3) was from urinary site and one isolate was related to ICU patient.

PFGE profile analysis enabled us to characterize a main clone referred as clone A, grouping 88.9% (n=24) isolates. Within this clone, it was observed 6 subclones (A1, A2, A3, A4, A5 and A6). The other three isolates were grouped into the clonal group B (Figure 1). All daptomycin-nonsusceptible isolates belonged to clone A, 60% (n=3) of them were grouped as subclone A6. The remaining two isolates were from subclones A2 and A4.

The isolate VRE_{fm}/11 was grouped into subclone A6 and it was susceptible to daptomycin by the two methodologies (BMD and Neo-Sensitabs prediffusion).

Discussion

VRE_{fm} has become one of the leading causes of nosocomial infection, especially among severely ill patients (Howden et al. 2013) and its dissemination is frequently clonal (Willems et al. 2005). Here, we described the susceptibility profile and the clonal relationship of 27 VRE_{fm} consecutively recovered from inpatients of a tertiary hospital in Southern Brazil, during one-year period. Among these isolates, we observed 18.5% of non-susceptibility to daptomycin. To our knowledge, this is the first description of daptomycin-nonsusceptible VRE_{fm} in Brazil.

A study in 2011 with isolates from 11 countries in Latin America determined antimicrobial susceptibility patterns for 218 enterococcis. The VRE percentage, considering all studied countries (Argentina, Brazil, Chile, Colombia, Costa Rica,

Ecuador, Guatemala, Mexico, Panama, Peru and Venezuela) was 14%. Brazil was the country with the highest rate of VRE (27%) (Jones et al. 2013). Moreover, as confirmed by our data and by other authors (Thongkoom et al. 2012; Wang et al. 2013; Deshpande et al. 2013), VRE_{fm} are historically associated to multidrug-resistance, severely reducing therapeutical options.

Around the world, studies have reported the spread of a genetic lineage of VRE_{fm} designated as clonal complex-17 (CC-17) by MLST (Brilliantova et al. 2010; Palazzo et al. 2011; López et al. 2012; da Silva et al. 2012). Isolates grouped into CC17 consistently exhibit resistance to ampicillin and ciprofloxacin. Besides, they can present a variable occurrence of resistance determinants, conferring multirresistance. They are very well adapted to the hospital environment (and exclusively associated to) and has been related with most of the reported hospital outbreaks worldwide (Willems et al. 2005; Panesso et al. 2010). Our isolates showed phenotypic characteristics similar to the CC17 lineage, which are ciprofloxacin and ampicillin resistance, but further studies must be developed to demonstrate if our VRE_{fm} belong to this specific lineage.

Although not approved by FDA for usage against glycopeptide-resistant enterococci, daptomycin is among the available therapeutical options for multiresistant VRE_{fm}. However, even though rare, some authors (and this work) around the world have been described non-susceptibility to daptomycin among enterococci, especially VRE_{fm}. In this context, Kelesidis (2011) did a systematic review including 23 studies that had cases of DNSE where 93.3% of the isolates were VRE, and among these, 88% were *E. faecium*. In a study conducted in Canada, among isolates of VRE_{fm} presenting resistance to ampicillin, ciprofloxacin and

linezolid, 33% were nonsusceptible to daptomycin. These authors performed MLST and confirmed that these isolates were genetically related to CC17 (Patel et al. 2013).

Daptomycin has a unique mechanism of action that targets the bacterial membrane and requires the presence of Ca^{2+} (Kelesidis et al. 2011). Although little is known about the mechanisms of resistance to this antimicrobial, some studies suggest that it is the ability of the membrane to change in response to stress and other stimuli (Palmer et al. 2011). Different factors have been discussed in the literature as the risk factors for the development of daptomycin resistance. Judge and co-workers (2012) conducted a case-control study with 60 cases of DNS-VRE versus daptomycin-susceptible VRE and noted that immunosuppression, multiple comorbidities, and prior exposures to cephalosporins and metronidazole were significant risk factor for the development of daptomycin resistance.

The emergence of resistance to daptomycin during therapy is worrisome and should be evaluated (Cattoir & Leclercq 2013). A study in Iowa City, USA, between 2005 and 2011 showed that the number of patients infected or colonized with DNSE was increasing and observed that most of the patients had prior exposure to daptomycin. Molecular typing revealed genetic heterogeneity among isolates, which possibly acquired this resistance phenotype due to the selective pressure (Storm et al. 2012). In contrast, we observed a high genetic similarity, and nonsusceptibility to daptomycin may be less linked to antimicrobial pressure, but associated to clonal spread. Indeed, this antibiotic is not used frequently in the referred hospital (average of 8 bottles/month of injectable administration in 1079 beds). This clonal spread may justify our considerably high rate of nonsusceptible isolates.

Some studies suggest that daptomycin resistance genes could have arisen in animals and would be transferred to humans via the food chain by the consumption

of food contaminated with DNSE isolates (Zhang et al. 2010; Kelesidis et al. 2012). However, more data are necessary to support this information.

Another point of concern is the methodology used to determine the non-susceptibility of enterococci to daptomycin. No agar diffusion methods are recommended by CLSI (2013). The committee recommends determination of MICs performing BMD with cation-adjusted Mueller-hinton broth (50µg/ml). Based on the mechanism of action of daptomycin, calcium concentration is a critical point and must be strongly considered. Etest, Neo-Sensitabs prediffusion and MicroScan are also methods available to determinate daptomycin susceptibility with different and sometimes questionable performances.

In a study conducted in North Carolina, among 50 isolates of *E. faecium* that presented daptomycin nonsusceptibility by Microscan method, six were nonsusceptible by Etest and only one isolate was categorized as having an MIC ≥8 ug/ml by BMD, reinforcing the difficulty to accurately determine this susceptibility profile. The authors suggest the use of more than one method for confirm results (Palavecino et al. 2013).

In one USA's institution it was noted a progressive decline in daptomycin susceptibility in E. *faecium* over the years 2008 to 2010 detected by MicroScan methods. One hundred and fifty DNSE recovered during the above cited period were tested by BMD and only 20% were defined as nonsusceptible, demonstrating that the prevalence of DNSE was overestimated by the first method (Bryant et al. 2013).

Katz and co-workers (2008) evaluated the results of BMD, Etest and Neo-Sensitabs prediffusion in isolates of *E. faecalis* and *E. faecium*, and observed that for *E. faecium* there are high variations of MICs among methodologies, suggesting that agar diffusion methods are not safe for this species. Indeed, the prediffusion also

overestimate our results, reinforcing the idea of combined methods to deal with this challenging phenotype

The major problem of different methodologies may be in calcium concentrations, particularly those using Mueller Hinton agar, since there may be ions variations between different lots of product and this may account for the heightened MICs obtained (Kelesidis et al. 2011).

Most VRE_{fm} of our study were clonal. This feature has been observed in studies conducted all around the world (Brilliantova et al. 2010; Panesso et al. 2010; López et al. 2012; Valdezate et al. 2012) and demonstrates the importance of infection control to prevent the spread of VRE_{fm} with such an impacting phenotype, as daptomycin non-susceptibility, and an extraordinary capacity to survive in hospital environment.

By comparing genome of our isolates with an isolate (VRE_{fm}/11) of a 2010/2011 outbreak which occurred in another hospital in the same city, we clearly observed a clonal relationship (same clone A), suggesting the spread of this well-established clone between different hospitals in Porto Alegre for a considerable long period, (2010 to 2013). The isolate VRE_{fm}/11 presented daptomycin-susceptibility and was grouped as subclone A6. This subclone also grouped 3 out of 5 isolates daptomycin-nonsusceptible.

Our study has some limitations. The clonal occurrence of VRE_{fm} is the major one, since it overestimates the rates of nonsusceptibility to daptomycin. Also, as we do not have access to patient's records, it was impossible to demonstrate a relationship among non-susceptible isolates and previous use of antimicrobials or other risk factors. Besides, deeper genetic analysis must be performed (MLST) to determine if Porto Alegre has been facing the CC17 lineage.

In conclusion, this is the first report of *E. faecium* nonsusceptible to daptomycin in Brazil, demonstrating the importance of systematic epidemiological studies to accompany and prevent dissemination of some specific and worrisome phenotypes/genotypes between health institutions of the city.

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References

Bryant KA, Roberts AL, Rupp ME, Anderson JR, Lyden ER, Fey PD, Van Schooneveld TC 2013. Susceptibility of enterococci to daptomycin is dependent upon testing methodology. Diagn Microbiol Infect Dis 76: 497-501.

Brilliantova NA, Kljasova GA, Miranova AV, Tishkov VI, Novichkova GA, Bobrynina VO, Sidorenko SV 2010. Spread of vancomycin-resistant *Enterococcus faecium* in two haematological centres in Russia. Int J Antimicrob Agents 35: 177-181.

Cattoir V, Leclercq R 2013. Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce?. Antimicrob Chemother. 68: 731–742.

Clinical and Laboratory Standards Institute (CLSI) (2013). Performance standards for antimicrobial susceptibility testing; twenty-third informational supplement M100-S23. Wayne, PA.

da Silva LP, Pitondo-Silva A, Martinez R, da Costa Darini AL 2012. Genetic features and molecular epidemiology of *Enterococcus faecium* isolated in two university hospitals in Brazil. Diagn Microbiol Infect Dis 74: 267-271.

d'Azevedo PA, Furtado GH, Medeiros EA, Santiago KA, Silbert S, Pignatari AC 2008. Molecular characterization of vancomycin-resistant Enterococci strains eight years apart from its first isolation in São Paulo, Brazil. Ver Inst Med Trop 50: 195-198.

Deshpande VR, Karmarkar MG, Mehta PR 2013. Prevalence of multidrug-resistant enterococci in a tertiary care hospital in Mumbai, India. J Infect Dev Ctries 7: 155-158.

Dutka-Malen S, Evers S, Courvalin P 1995. Detection of glicopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol 33: 24-27.

Howden BP, Holt KE, Lam MM, Seemann T, Ballard S, Coombs GW, Tong SY, Grayson ML, Johnson PD, Stinear TP 2013. Genomic insights to control the emergence of vancomycin-resistant enterococci. MBio 4: 1-9.

Jones RN, Guzman-Blanco M, Gales AC, Gallegos B, Castro AL, Martino MD< Vega S, Zurita J, Cepparulo M, Castanheira M 2013. Susceptibility rates in Latin American nations: report from a regional resistance surveillance program (2011). Braz J Infect Dis 17: 672-681.

Judge T, Pogue JM, Marchaim D, Ho K, Kamatam S, Parveen S, Tiwari N, Nanjireddy P, Bheemreddy S, Biedron C, Reddy SM, Khammam V, Chalana IK, Tumma RS, Collins V, Yousuf A, Lephart PR, Martin ET, Rybak MJ, Kaye KS, Hayakawa K 2012. Epidemiology of vancomycin-resistant enterococci whit reduced susceptibility to daptomycin. Infect Control Hosp Epidemiol 33: 1250-1254.

Katz BD, Luperchio AS, Thorne GM 2008. Detection of daptomycin-nonsusceptible strains using the Neo-Sensitab prediffusion method. Diagn Microbiol Infect Dis 61: 315-320.

Kariyama R, Mitsuhata R, Chow JW, Clewel DB, Kumon H 2000. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J Clin Microbiol 38: 3092-3095.

Kelesidis T, Humphries R, Uslan DZ, Pegues DA 2011. Daptomycin nonsusceptible enterococci: an emerging challenge for clinicians. Clin Infect Dis. 52: 228-234.

Kelesidis T, Humphries R, Uslan DZ, Pegues DA 2012. De novo daptomycin-nonsuscetible enterococcal infections. Emerg Infect Dis. 18: 674-676.

Lebreton F, van Schaik W, McGuire, AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJL, Earl AM, Gilmore MS 2013. Emergence of Epidemic Multidrug-Resistant *Enterococcus faecium* from Animal and Commensal Strains. mBio, 4: 1-10.

López M, Cercenado E, Tenorio C, Ruiz-Larrea F, Torres C 2012. Diversity of clones and genotypes among vancomycin-resistant clinical *Enterococcus* isolates recovered in a Spanish hospital. Microb Drug Resist, 18: 484-491.

Magiorakos AP, Srinivasan A, Carey RB, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liliequist B, Paterson DL, Rice LB, Stelling J, Struelens

MJ, Vatopoulos A, Weber JT, Monnet DL 2012. Multidrug-resistant, extensively, drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18: 268-281.

Mohr JF, Friedrich LV, Yankelev S, Lamp KC 2009. Daptomycin for the treatment of enterococcal bacteraemia: results from the Cubicin outcomes registry and experience (CORE). Int J Antimicrob Agents 33: 543-548.

Mondino SS, Castro AC, Mondino PJ, Carvalho MGS, Silva KMF, Teixeira LM 2003. Phenotypic and genotypic characterization of clinical and intestinal enterococci isolated from inpatients and outpatients in two Brazilian hospitals. Microb Drug Resist 9: 167-174.

Panesso D, Reyes J, Rincón S, Díaz L, Galloway-Peña J, Zurita J, Carrillo C, Merentes A, Guzmán M, Adachi JA, Murray BE, Arias CA 2010. Molecular epidemiology of vancomycin-resistant *Enterococcus faecium*: a prospective, multicenter study in South American hospitals. J Clin Microbiol 48: 1562-1569.

Palazzo IC, Pitondo-Silva A, Levy CE, da Costa Darini AL 2011. Changes in vancomycin-resistant *Enterococcus faecium* causing outbreaks in Brazil. J Hosp Infect 79: 70-74.

Palavecino EL, Burnell JM 2013. False daptomycin-nonsusceptible MIC results by Microscan panel 29 relative to Etest results for *Staphylococcus aureus* and enterococci. J Clin Microbiol 51: 281-283.

Palmer KL, Daniel A, Hardy C, Silverman J, Gilmore MS 2011. Genetic Basis for Daptomycin Resistance in Enterococci. Antimicron Agents Chemother 55: 3345-3356.

Patel SN, Memari N, Shahinas D, Toye B, Jamieson FB, Farrell DJ 2013. Linezolid resistance in *Enterococcus faecium* isolated in Ontario, Canada. Diagn Microbiol Infect Dis x, xxx-xxx.

Resende M, Caierão J, Prates JG, Narvaez GA, Dias CA, d'Azevedo PA 2014. Emergence of *vanA* vancomycin-resistant *Enterococcus faecium* in a hospital in Porto Alegre, South Brazil. J Infect Dev Ctries 8: 160-167.

Storm JC, Diekema DJ, Kroeger JS, Johnson SJ, Johannsson B 2012. Daptomycin exposure precedes infection and/or colonization whit daptomycin nonsusceptible *Enterococcus*. Antimicrob Resist Infect Control 1: 1-7.

Teixeira LM, Carvalho MG, Merquior VL, Steigerwalt AG, Teixeira MG, Brenner DJ, Facklam RR 1997. Recent approaches on the taxonomy of the enterococci and some related microorganisms. Adv Exp Med Biol 418: 397-400.

Tran TT, Panesso D, Mishra NN, Mileykovskaya E, Guan Z, Munita JM, Reyes J, Diaz L, Weinstock GM, Murray BE, Shamoo Y, Dowhan W, Bayer AS, Arias CA 2013. Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. MBio 23: 1-10.

Teixeira LM, Carvalho MGS, Shewmaker PL, Facklam RR 2011. *Enterococcus.* In: Versalovic, J.; Carrol, K.C.; Funke, G.; Jorgensen, J.H.; Landry, M.L.; Warnock, D.W. Manual of Clinical Microbiology, 10th ed., v. 1, American Society for Microbiology, Washington, DC, p. 350-364.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33: 2233-2239.

Thongkoom P, Kanjanahareutai S, Chantrakooptungool S, Rahule S, Zhang J, Wall SK, Xu L, Ebner PD 2012. Vancomycin-resistant enterococci (VRE) isolates isolated in Rajavithi Hospital between 1999 and 2009. J Med Assoc Thai 95: S7-15.

Valdezate S, Miranda C, Navarro A, Freitas AR, Cabrera JJ, Carrasco G, Coque TM, Jiménez-Romano E, Saéz-Nieto JA 2010. Clonal outbreak of ST17 multidrug-resistant *Enterococcus faecium* harbouring an Inc18-like: Tn1546 plasmid in a haemo-oncology ward of a Spanish hospital. J Antimicrob Chemother 67: 832-836.

Zhang J, Wall SK, Xu L, Ebner PD 2010. Contamination Rates and Antimicrobial Resistance in Bacteria Isolated from “Grass-Fed” Labeled Beef Products. Foodborne Pathog Dis 7: 1331-1336.

Wang JT, Chang SC, Wang HY, Chen PC, Shiau YR, Lauderdale TL 2013. High rates of multidrug resistance in *Enterococcus faecalis* and *E. faecium* isolated from inpatients and outpatients in Taiwan. Diagn Microbiol Infect Dis 75: 406-411.

Willems RJ, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJM 2005. Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. Emerg Infect Dis 11: 821-828.

Willems RJ, van Schaik W 2009. Transition of *Enterococcus faecium* from commensal organism to nosocomial pathogen. Future Microbiol 4: 1125-1135.

Table 1. Description of primers used in PCR for the detection of species and vancomycin-resistance genes of *Enterococcus faecium*.

Target gene	Sequence of primer	Amplicon Size (pb)	Reference
Enterococcus faecium	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	658	Karyama *et al.*, 2000.
Enterococcus faecalis	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	941	Karyama *et al.*, 2000.
vanA	5'-CATGAATAGAATAAAAGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	1030	Karyama *et al.*, 2000.
vanB	5’-GTGACAAACCGGAGGCGAGGA-3’ 5’-CCGCCATCCTCCTGCAAAAAA-3’	433	Karyama *et al.*, 2000.

Table 2. Results of antimicrobial susceptibility testing, date of isolation and clonal type of 27 strains of *Enterococcus faecium* isolated from different clinical materials.

Isolate	Antimicrobial Agent[a]										Isolation Site	Date	Origin[d]	Clonal Type
	AMP	HLR-Ge	VAN	TEI	Q/D	LIN	CIP	NIT	DAP[b]	DAP[c]				
10	R	S	R	R	S	S	R	-	S	NS	Blood	September/12	ICU	A3
51	R	S	R	R	S	S	R	R	NS	NS	Urine	October/12	Other	A6
75	R	S	R	R	S	S	R	-	S	NS	Blood	October/12	ICU	B1
97	R	S	R	R	S	S	R	R	S	NS	Urine	October/12	Other	A3
109	R	S	R	R	S	S	R	-	S	NS	Blood	November/12	ICU	A2
127	R	S	R	R	S	S	R	-	S	NS	Blood	December/12	ICU	A1
128	R	S	R	R	S	S	R	-	S	NS	Blood	December/12	ICU	A4
217	R	R	R	R	S	S	R	R	S	NS	Urine	February/13	Other	A1
218	R	S	R	R	S	S	R	-	NS	NS	Pleural Fluid	February/13	ICU	A6
237	R	S	R	R	S	S	R	R	NS	NS	Urine	March/13	Other	A4
253	R	R	R	R	S	S	R	R	S	NS	Urine	April/13	Other	A4
254	R	S	R	R	S	S	R	-	S	NS	Catheter	April/13	Other	A2
284	R	S	R	R	S	S	R	-	S	NS	Blood	May/13	ICU	A4
286	R	S	R	R	S	S	R	-	NS	NS	Blood	May/13	Other	A2
299	R	S	R	R	S	S	R	-	S	NS	Abdominal secretion	May/13	ICU	A2
315	R	S	R	R	S	S	R	-	S	NS	Blood	May/13	ICU	A5
324	R	S	R	R	S	S	R	R	NS	NS	Urine	May/13	Other	A6
326	R	S	R	R	S	S	R	-	S	NS	Blood	May/13	Other	A2
362	R	S	R	R	S	S	R	R	S	NS	Urine	June/13	Other	B1
380	R	S	R	R	S	S	R	-	S	NS	Blood	July/13	Other	B1
385	R	R	R	R	S	S	R	-	S	NS	Blood	July/13	ICU	A4
401	R	S	R	R	S	S	R	R	S	NS	Urine	July/13	Other	A5
402	R	S	R	R	S	S	R	-	S	NS	Stool	July/13	Other	A3
427	R	S	R	R	S	S	R	-	S	-	Abdominal secretion	August/13	ICU	A2
428	R	S	R	R	S	S	R	-	S	-	Blood	August/13	ICU	A2
430	R	R	R	R	S	S	R	R	S	NS	Urine	August/13	ICU	A1
431	R	S	R	R	S	S	R	-	S	-	Stool	August/13	Other	A1
VRE_{fm} 2011	R	S	R	R	S	S	R	-	S	S	Urine	September/11	Other hospital	A6

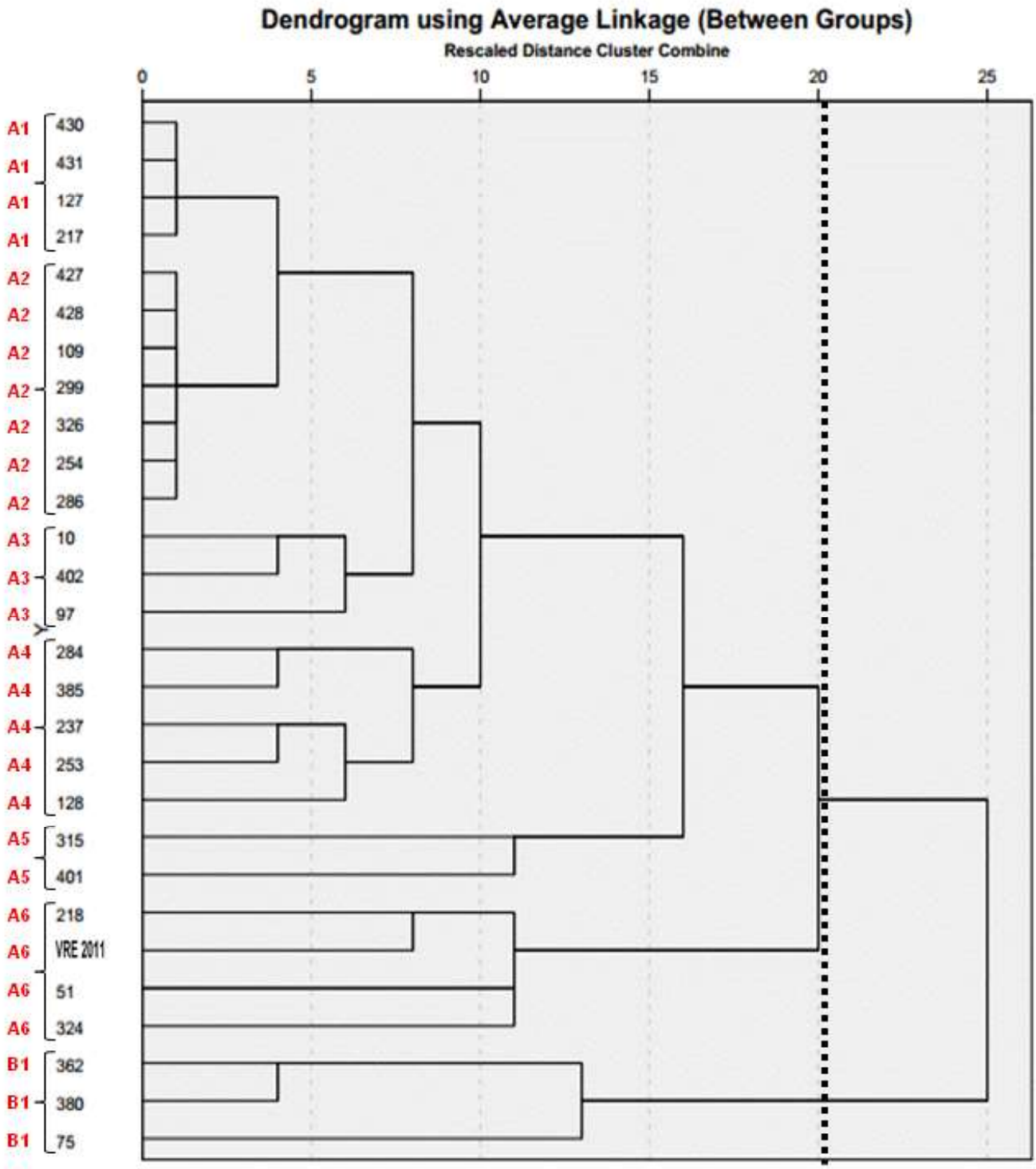
[a] AMP – ampicillin; HLR-Ge – high levels resistance to gentamicin; VAN – vancomycin; TEI – teicoplanin; Q/D - quinupristin-dalfopristin; LIN – linezolid; CIP – ciprofloxacin; NIT – nitrofurantoin; DAP – daptomycin.

[b] determined by broth microdilution method

[c] determined by Neo-sensitabs prediffusion

[d] ICU (Intensive Care Unit)

Figure 1. Dendogram showing similarity among *E. faecium* isolates from different patients.



5. CONCLUSÕES

Os resultados obtidos na presente dissertação através das características fenotípicas e genotípicas dos enterococos estudados permitem concluir:

- *Enterococcus faecalis* foi a espécie mais frequentemente isolada em amostras clínicas, principalmente em infecções do trato urinário. Entretanto, *E. faecium* vem emergindo no hospital estudado.
- *Enterococcus faecium* é mais resistente a maioria dos antimicrobianos testados, e como observado, na maioria das vezes é clonal e apresenta características semelhantes a outros isolados descritos em estudos anteriores nesta cidade e semelhante a isolados reportados em diferentes partes do mundo, demonstrando a importância de se desenvolver estratégias adequadas para evitar a disseminação desse micro-organismo. Este estudo foi o primeiro a reportar casos de não-susceptibilidade a daptomicina no Brasil, demonstrando a importância de acompanhar esses novos fenótipos de resistência que vem emergindo, como forma de evitar a disseminação.
- A capacidade para formar biofilme é forte em *Enterococcus*, principalmente em isolados de *E. faecali*s oriundos de sítios urinários.
- *Enterococcus faecalis* apresentou mais fatores de virulência e todos foram significativamente associados com a formação de biofilme nessa espécie. Já *E. faecium* apresentou menos fatores de virulênca, porém, como os fatores estudados foram originalmente achados em *E. faecalis*, *E. faecium* pode carrear outros fatores que ainda pouco se conhece.

6. ANEXOS

6.1 Aprovação do Comitê de Ética em Pesquisa da UFCSPA

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Avaliação da capacidade de formação de biofilme e detecção dos genes Esp, Espefm e GelE em isolados clinicos de Enterococcus faecalis E e Enterococcus faecium.

Pesquisador Responsável Pedro Alves d'Azevedo Parecer 1730/12

Data da Versão 09/04/2012 | Cadastro 967/12 | Data do Parecer 21/06/2012

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

Objetivos do Projeto
Analisar a capacidade de formação de biofilmes entre os isolados clínicos de Enterococcus faecalis e Enterococcus faecium.

Sumário do Projeto
Para efetuar os ensaios de formação dos biofilmes serão utilizados 200 amostras de isolados clinicos de Enterococcus faecalis e Enterococcus faecium. Serão utilizados na pesquisa exclusivamente isolados bacterianos. Em nenhuma das etapas do projeto será empregado material biológico humano, conforme descrito na Resolução 441.

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
Claros, objetivos e factíveis de serem respondidos.

Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total 200 Local
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Não
Seleção eqüitativa dos indivíduos participantes	Adequada
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Adequada
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	Adequado
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Cronograma	Adequado
Data de início prevista	
Data de término prevista	
Orçamento	Adequado

Fonte de financiamento externa	Não

Comentários sobre o Cronograma e o Orçamento

Referências Bibliográficas	Adequadas

Comentários sobre as Referências Bibliográficas
atual e pertinente para a pesquisa

Recomendação
Aprovar

Comentários Gerais sobre o Projeto

6.2 Aprovação do Comitê de Ética em Pesquisa da ISCMPA

IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE - ISCMPA

Plataforma Brasil

PROJETO DE PESQUISA

Título: Avaliação da Capacidade de Formação de Biofilme e Detecção dos Genes Esp, Espefm e GelE em isolados clínicos de Enterococcus faecalis e Enterococcus faecium

Área Temática:

Versão: 1

CAAE: 03782312.2.0000.5335

Pesquisador: Renata Oliveira Soares

Instituição: IRMANDADE DA SANTA CASA DE MISERICORDIA DE PORTO ALEGRE (PAVILHAO PEREIRA FILHO)

PARECER CONSUBSTANCIADO DO CEP

Número do Parecer: 90.409

Data da Relatoria: 04/09/2012

Apresentação do Projeto:

O projeto procura estudar a capacidade de formação e biofilme por Enterococcus faecalis e Enterococcus faecium isolados em pacientes internados na Santa Casa de Misericórdia.Para isto estudará genes dos microorganismos: Esp, Espefm e GelE. As amostras serão obtidas de exames solicitados ao Laboratório Central da Santa Casa.

Objetivo da Pesquisa:

O objetivo do estudo é avaliar a capacidade de formação de biofilme dos germes isolados em pacientes internados e verificar se são mais ativos que os germes fora do hospital.

Avaliação dos Riscos e Benefícios:

O estudo não realiza intervenção em pacientes. Utilizará germes identificados em culturas solicitadas pelos médicos assistentes ao Laboratório Central. Utilizará dados de prontuário do paciente. Não envolve riscos para os pacientes. Não trará benefícios para os pacientes que forneceram o material, entretanto poderá ser útil para futuros pacientes.

Comentários e Considerações sobre a Pesquisa:

A pesquisa será feita com microorganismos obtidos junto ao Laboratório Central a partir de amostras de pacientes internados.Trata-se de pesquisa em laboratório. Não apresenta riscos para os pacientes.

Considerações sobre os Termos de apresentação obrigatória:

Apresenta orçamento e financiamento
Apresenta declaração de confidencialidade e de utilização dos dados.
Apresenta cronograma.
Não haverá ônus para a instituição.

Recomendações:

Não se aplica.

Conclusões ou Pendências e Lista de Inadequações:

O presente projeto respeita as normas existentes para pesquisa envolvendo seres humanos. Nosso parecer é favorável a sua aprovação.

O presente projeto respeita as normas existentes para pesquisa envolvendo seres humanos. Nosso parecer é favorável a sua aprovação.

Situação do Parecer:

Aprovado

6.3 Trabalhos apresentados em Eventos Científicos

- **27º Congresso Brasileiro de Microbiologia**
  Natal/RN – Brasil

Resumo: Avaliação da capacidade de formação de biofilme associada com a presença dos genes *gelE*, *esp* e *agg* em isolados clínicos de *Enterococcus faecalis* e *Enterococcus faecium*

Soares, Renata Oliveira; Fedi, Ana Cláudia; Reiter, Keli Cristine; Caierão, Juliana; d'Azevedo, Pedro.

- **16º Encontro Nacional de Biomedicina**
  Botucatu/SP – Brasil

Resumo: Avaliação do perfil de suscetibilidade antimicrobiana de *Enterococcus faecalis* e *Enterococcus faecium* isolados em um hospital de Porto Alegre - RS

Soares, Renata Oliveira; Fedi, Ana Cláudia; Caierão, Juliana; d'Azevedo, Pedro.

- **4th ASM Conference on Enterococci**
  Cartagena – Colombia

Abstract: First report of Daptomycin-nonsusceptible *Enterococcus faecium* isolated in Brazil

Soares, R.O.; Batista, B.G.; Fedi, A.C.; Reiter, K.C.; Caierão, J.; d'Azevedo, P.A.

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Virulence recognizes that excellent papers may have been erroneously rejected by other journals. We will reconsider papers that have been rejected by *Nature, Science, Nature Medicine, Nature Cell Biology, Cell, Cancer Cell, Cell Metabolism, Developmental Cell, NEJM, Lancet, Genes & Development* and some other journals in the original format of those journals, thus saving the authors effort and time. Authors are encouraged to enclose the reviewers' and/or editorial comments from the journals mentioned above. This will expedite the evaluation of the article. In some instances, the article may be accepted based on the previous review. This allows urgent and competitive research to be published soon after submission. Papers submitted using the Select Submission Track can be accepted within 1-2 days.

Please submit your paper and contact the Editor-in-Chief with the manuscript number and a PDF with the previous review. Upon acceptance, authors should provide the paper in *Virulence*'s format.

Pre-submission Inquiries

Pre-submission inquiries are not necessary but are welcome. These may include either an abstract or a full-length manuscript as an email attachment (Microsoft Word). Pre-submission inquiries should be emailed to the Editor-in-Chief (Eleftherios Mylonakis).

Non-Native Speakers of English

Authors who are not native speakers of English and submit manuscripts to international journals, often receive negative comments from referees or editors about English-language usage. These problems can contribute to a decision to reject a paper. To help reduce the possibility of such problems, we strongly encourage such authors to take at least one or both of the following steps:

- Have your manuscript reviewed for clarity by a colleague whose native language is English.
- Use a service such as one of those listed at the end of our guidelines. An editor will improve the English to ensure that your meaning is clear and identify problems that require your review. Note that the use of such a service is at the author's own expense and risk, and does not guarantee that the article will be accepted. Landes Bioscience accepts no responsibility for the interaction between the author and the service provider or for the quality of the work performed.

Manuscript Preparation

Types of Papers

Research Papers/Reports
Research Papers or Reports should include the following sections in the following order:

- Abstract: A single paragraph of fewer than 250 words. The primary goal of the abstract should be to make the general significance and conceptual advance of the work clearly accessible to a broad readership. References should not be cited in the abstract.
- Introduction.
- Results: Present results in a logical sequence in tables and illustrations. In the text, explain, emphasize or summarize the most important observations. Units of measurement should be expressed in accordance with Systeme International d'Unites (SI Units).
- Discussion: Do not repeat in detail data given in the Results section. Emphasize the new and important aspects of the study. Relate observations to other relevant studies. On the basis of your findings (and others'), discuss possible implications/conclusions. When stating a new hypothesis, clearly label it as such.
- Patients and Methods/Materials and Methods: Describe the selection of patients or experimental animals, including controls. Do not use patients' names or hospital numbers. Identify methods, apparatus (manufacturer's name and address) and procedures in sufficient detail to allow other workers to reproduce the results. Provide references and brief descriptions of methods that have been published. When using new methods, evaluate their advantages and limitations. Identify drugs and chemicals, including generic name, dosage and route(s) of administration.
- Indicate whether the procedures were approved by the Ethics Committee of Human Experimentation in your country, or are in accordance with the Helsinki Declaration of 1975.
- For reagents listed in the Materials and Methods section, the company that supplied the reagent and the catalog number should be listed in parentheses; do not list the company location.
- References: No more than 85.
- Figure legends.
- Tables: Tables should be numbered consecutively with Arabic numerals and include descriptive titles and legends.

Reviews

Reviews should be recognized as scholarly by specialists in the field being covered, but should also be written with a view to informing readers who are not specialized in that particular field, and should therefore be presented using simple prose. Please avoid excessive jargon and technical detail. Reviews should capture the broad developments and implications of recent work. The opening paragraph should make clear the general thrust of the review and provide a clear sense of why the review is now particularly appropriate. The concluding paragraph should provide the reader with an idea of how the field may develop or future problems to overcome, but should not summarize the article. To ensure that a review is likely to be accessible to as many readers as possible, it may be useful to ask a colleague from another discipline to read the review before submitting it. Submitted reviews are subject to the same page charges as full-length reports—whether and how page charges will apply for commissioned reviews will be determined upon each commission. Reviews should include an abstract of 150 words and should cite no more than 150 references. Please include 5-10 key words for indexing purposes.

Protocols

Protocols describe a powerful experiment, a method or important technical updates relevant to the field. A high priority of *Virulence* Protocols is that all protocols be accessible to nonspecialists, thus the text and figures should be readable and clear to those outside the field. The Protocols section only features proven protocols, thus the authors of the protocol must have previously used their method to produce the work reported in a peer-reviewed primary journal.

Generally Protocols are commissioned by the editorial team, but pre-submission inquiries are also welcome. Commissioned protocols will undergo peer review, thus commissioning does not guarantee publication.

Please include the following:

- Title: (should describe the method and, if appropriate, its application).
- Abstract: (~150 words). Should briefly describe the protocol, its applications and the results that can be expected. If possible, include a final sentence indicating how long the whole protocol takes.
- Introduction: Should enable readers to make a decision as to the suitability of the protocol to their experimental problem. You should introduce the technique under discussion and include references to key papers where the protocol has been used previously. This section should include information on (a) the development of the protocol, (b) potential applications of the method, (c) comparison with other methods and (d) experimental design with procedure-specific information.
- Materials: Please use the subheadings (a) Reagents, (b) Equipment, (c) Reagent Setup and (d) Equipment Setup to structure this section. Subheadings (c) and (d) are optional.
  a. Reagents: List of essential reagents, including information about the suppliers. If you have found that deviations from a particular reagent, or its source, have adverse effects on the outcome of the protocol this should be made clear. Toxic or harmful agents should be highlighted, followed by a brief explanation of the hazard and the precautions that should be taken when handling the agent.
  b. Equipment: List essential equipment, highlighting any specialist equipment required.
  c. Reagent Setup: This section is appropriate for including details on required sample specifications (i.e. minimum protein quantity and allowed buffer components), preparation of a complicated buffers, the pre-treatment of solvents and/or reagents to make sure they are moisture-free and/or air-free. Please indicate whether a buffer/solution should be made up fresh or can be stored and, if so, under which conditions and for how long. In addition please state whether % solutions are wt/vol or vol/vol.
  d. Equipment Setup: This section should contain details on the setup of equipment (i.e. HPLC separation methods).
- Procedure: This step by step methodology is the major part of the protocol and must be a numbered list. If the protocol naturally breaks into separate stages, then include subheadings and resume the numbered list. State how long the section will take to complete. Include diagrams and/or photographs of equipment set-up, where appropriate. If the protocol is complicated, you should consider including a flow diagram to demonstrate how the stages fit together. Please include PROBLEM callouts after steps where problems are encountered, that are subsequently mentioned in the Problem Handling section.
- Timing: If possible, please include a timeline indicating the approximate time a step, or set of steps, will take. Provide this information as a summary at the end of the procedure.
- Problem Handling (Problem Tracing, Fault Finding, Fault Diagnosis): Include information on how to trace and correct the most likely problems users will encounter with the protocol. Please provide this information in the form of a table with the columns 'Step', 'problem', 'possible reason', 'solution'. The step number should be given where the problem is first observed (not where it occurred). The appropriate steps should also be flagged in the main text by the heading 'Problem'. If problem handling text refers to only one or two steps, it can also be formatted as normal text with subheadings referring to the steps or sections that the information pertains to.
- Anticipated Results: Include information about the likely outcome for users, for example, likely yield of protein, typical microscopy images, etc. This is a good place to include directions on how to interpret and analyze the raw data, including equations if necessary.

Brief Reports

These are short experimental papers that present unusually interesting data combined with a discussion of what the data might mean, or an explanation of why the data contradicts current paradigms. The primary goal of the abstract should be to make the general significance and conceptual advance of the work clearly accessible to a broad readership.

The length of a Short Paper should not exceed 15 double-spaced printed manuscript pages of text inclusive of references and exclusive of Figures and Tables.

Short papers should include the following sections:

1. Abstract: maximum 150 words
2. Keywords: 5-10 for indexing purposes
3. References: maximum 30

News & Views
News & Views are essentially auto-commentary. The Editorial Board will solicit authors of the most significant recent and forthcoming papers, published elsewhere, to provide a short summary with additional insights, new interpretations or speculation on the relevant topic. The News & Views should not include data, but model figures are acceptable; if you wish to include unpublished data, please use the Brief Report format. The first paragraph will constitute the abstract (50–75 words) and the text is limited to 1,000-3,000 words (not including figure legends, which should be a maximum of 150 words). News & Views will have no references, but the original paper will still be noted on the first page.

Perspectives
Perspectives may be short and focused opinion articles, commentaries on papers recently published in *Virulence* or elsewhere, or commentaries on significant conceptual changes, important trends or new directions in the field. These may include figures and up to 30 references. Please include an abstract of 150-200 words and 5-10 key words for indexing purposes.

Meeting Reports
Meeting Reports are summaries of presentations from recent meetings in the field. Authors are encouraged to contact the Editor-in-Chief with proposals for meeting reports. Also, please contact the meeting organizers to verify that reports will be permitted. Please include an abstract of 150-200 words and 5-10 key words for indexing purposes.

Letters to the Editor
Letters to the Editor are aimed at publishing short, but important, breakthrough data not embedded within a complex story. This can also be what is considered a Small Publishable Unit. In other words, data that is sufficient in itself to be published, but not a part of a larger story that would comprise an entire research article.

Letters to the Editor can also be mini-reviews with an small addition of novel data.

The abstract should not be longer than 120 words. The paper should be structured as a research paper (see above), but without the headings and subheadings. No more than 50 references and no more than 4,500 words altogether.

Organization

All manuscripts should be in English. Please ensure that manuscripts are clear, concise and grammatically correct.

Text should be prepared in MS Word, double-spaced, with page numbers throughout and line numbering turned on. Click here for directions on adding line numbers.

Organize manuscripts in the following manner:

- Title page, including titles, author's names (first, MI, last) and affiliations
- 5-10 keywords (for indexing purposes)
- A list of abbreviations and acronyms used throughout the text
- An abstract (please see Type of Paper for word limit), the primary goal of which is to make the general significance and conceptual advance of the work clearly accessible to a broad readership. (References should not be cited in the abstract.)
- Text (length and organization depends upon type of paper)
- Acknowledgments
- References
- Figure legends
- Tables (with descriptive titles and legends)

There are no word limits for papers published, however, accepted manuscripts are published with the understanding that page and color charges will be assessed. Please see the section, Page and Color Charges below.

If your paper is to be published in a journal indexed by PubMed/Medline, the citation of your article will be sent to PubMed within one week of acceptance; therefore, please ensure that all information is correct.

Text Files/Tables

Please save text and table files as MS Word documents. Figure legends should be at the end of the manuscript following references. Tables will be reformatted during production and therefore should only be minimally formatted in your text file and follow the figure legends.

Figure Preparation

Figures should be as small and simple as clarity permits. Unnecessary figures and panels in figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Avoid unnecessary complexity, coloring and excessive detail. Figures should not contain more than one panel unless the parts are logically connected. Where possible, text, including keys to symbols, should be provided in the text of the figure legend rather than on the figure itself. Any image processing should be explained clearly in the Materials and Methods section of your manuscript.

To aid in the processing and turnaround of issues, we ask that authors please adhere to the following figure guidelines. Authors will be asked to revise details and images if they do not adhere to the figure protocols.

Guidelines for Figure Preparation

Image presentation

(These guidelines for image presentation are adapted from the "Instructions for Authors" that are posted on the *Journal of Cell Biology* web site, and are included here with permission).

As you prepare your figures, please adhere to the following guidelines to accurately present your data:

1. No specific feature within an image may be enhanced, obscured, moved, removed, or introduced.
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A more detailed discussion of image presentation can be found at the following URL: http://jcb.rupress.org/content/166/1/11.full (Rossner and Yamada, J. Cell Biol. 166:11–15)

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All submitted images must be of high quality and have resolutions of 300 dpi ready for print.

Formats

We require figures in electronic format. Please do not send PowerPoint, MS Word, presentation or paint files as they are inadequate for the creation of high quality images. Much of the information contained in PowerPoint or other file types is lost or skewed in the conversion of images. Figures should be provided as TIF, Photoshop, EPS or high resolution PDF files. Compatible graphic art programs are Adobe Illustrator and Adobe Photoshop.

Figure size

Figures should be submitted at the size they are to be published. Maximum width = 7.1 in. Maximum height = 9.5 in.

For multi-panel figures (such as figure 1a, 1b, 1c, etc.), each panel should be assembled into one image file. Do not include separate panels on multiple pages, i.e. A, B, C and D should all fit on one page. Each panel should be sized so that the figure as a whole can be reduced by the same amount and reproduced on the printed page at the smallest size at which essential details, including type, are visible and readable.

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Save all color figures in CMYK mode at 8 bits/channel. Layering type directly over shaded or textured areas and using reversed type (white lettering on a colored background) should be avoided.

Type

Please be sure to embed all fonts. Use a sans serif font such as Helvetica. The font size should be no greater than 9 pt. and no smaller than 6 pt; however, panel labels (A, B, C) should be 15 pt. uppercase (not bold). Lettering in figures (labeling of axes and so on) should be in lowercase type, with the first letter capitalized and no full stop. Please keep font size relatively the same throughout the figures so as to avoid scaling issues. Also note that readability suffers if type is layered over a pattern or color other than white or black.

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Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by commas (1,000). Unusual units or abbreviations should be defined in the legend. Please use the proper microsymbol (denoting a factor of one millionth) rather than a lower case u.

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Use the citation-sequence system: The list of references should be numbered consecutively according to the sequence of first appearance within the article text. For in-text references, use only the number assigned to the reference:

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Correct: For a review see refs. 20-25.
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Journal References

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- Abbreviate journal names according to the style used in Index Medicus or a comparable source and omit punctuation after journal titles. Spell out foreign or less commonly known journal names.

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Other Types of References
For all other types of reference styling formats, please refer to the National Library of Medicine Style Guide for Authors, Editors and Publishers which is available here: http://www.ncbi.nlm.nih.gov/books/NBK7256

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All potential cover images should be sized to fit on a single letter size (8.5" x 11") page. Please remove all text, captions, etc. from the image. If you have variations of the image, you may send additional files. Please send no more than two alternate versions.

Accepted formats and resolution:

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- TIF, 300 dpi, CMYK at 100% size
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Objetivos e política editorial

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Formato e estilo

O manuscrito (incluindo tabelas e referências) deve ser preparado em um software para edição de textos, em espaço duplo, fonte 12, paginado. As margens devem ser de pelo menos 3 cm. As figuras deverão vir na extensão tiff, com resolução mínima de 300 dpi. Tabelas e figuras deverão vir em documentos separados.

Deve ser organizado de acordo com a seguinte ordem:

Título resumido: com até 40 caracteres (letras e espaços)

Título: com até 250 caracteres

Autores: sem títulos ou graduações

Afiliação institucional: endereço completo somente do autor correspondente

Resumo: com até 200 palavras (100 palavras no caso de comunicações breves). Deve enfatizar novos e importantes aspectos do estudo ou observações.

Palavras-chave: devem ser fornecidos de 3 a 6 termos, de acordo com a lista Medical Subject Headings (Mesh) do Index Medicus.

Notas de rodapé: indicando a fonte de financiamento e mudança de endereço

Introdução: deve determinar o propósito do estudo, oferecer um breve resumo (e não uma revisão de literatura) dos trabalhos anteriores relevantes, e especificar quais novos avanços foram alcançados através da pesquisa. A introdução não deve incluir dados ou conclusões do trabalho em referência.

Materiais e Métodos: deve oferecer, de forma breve e clara, informações suficientes para permitir que o estudo seja repetido por outros pesquisadores. Técnicas padronizadas bastam ser referenciadas.

Ética: ao descrever experimentos relacionados a temas humanos, indicar se os procedimentos seguidos estiveram de acordo com os padrões éticos do comitê responsável por experimentos humanos (institucional ou regional) e de acordo com a Declaração de Helsinki de 1975, revisada em 1983. Ao relatar experimentos em animais, indicar se diretrizes de conselhos de pesquisa institucionais ou nacionais, ou qualquer lei nacional relativa aos cuidados e ao uso de animais de laboratório foram seguidas.

Resultados: devem oferecer uma descrição concisa das novas informações descobertas, com o mínimo julgamento pessoal. Não repetir no texto todos os dados contidos em tabelas e ilustrações.

Discussão: deve limitar-se ao significado de novas informações e relacionar as novas descobertas ao conhecimento existente.

Somente as citações indispensáveis devem ser incluídas.

Agradecimentos: devem ser breves e concisos e se restringir ao absolutamente necessário.

Referências: devem ser precisas. Somente as citações que aparecem no texto devem ser referenciadas. Trabalhos não publicados, a não ser os já aceitos para publicação, não devem ser citados. Trabalhos aceitos para publicação devem ser citados como " in press "; nesse caso, uma carta de aceitação da revista deverá ser fornecida. Dados não publicados devem ser citados somente no texto como " unpublished observations "; nesse caso, uma carta com a permissão do autor deve ser fornecida. As referências ao final do manuscrito devem ser organizadas em ordem alfabética de acordo com o sobrenome do primeiro autor.

Os títulos de revistas devem ser abreviados de acordo com o estilo usado no Index Medicus. Consultar:
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• **No texto, usar o sobrenome do autor e a data**:
Lutz (1910) ou (Lutz 1910).

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• **Nas referências, usar os seguintes estilos**:
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Forattini OP 1973. Entomologia Médica. Psychodidae, Phlebotominae, Leishmaniose, Bartonelose, Vol. IV, Edgard Blucher, São Paulo, 658 pp.

Morel CM 1983. Genes and Antigens of Parasites. A Laboratory Manual, 2nd ed., Fundação Oswaldo Cruz, Rio de Janeiro, xxii + 580 pp.

Mello-Silva CC 2005. Controle alternativo e alterações fisiológicas em Biomphalaria glabrata (Say, 1818), hospedeiro intermediário de Schistosoma mansoni Sambom, 1907 pela ação do látex de Euphorbia splendens var. hislopii N.E.B (Euphorbiaceae), PhD Thesis, Universidade Federal Rural do Rio de Janeiro, Seropédica, 85 pp.

Capítulo de livro
Cruz OG 1911. The prophylaxis of malaria in central and southern Brasil. In R Ross, The Prevention of Malaria, John Murray, London, p. 390-398.

Artigo de revista na Internet
Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12];102(6):[about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

Monografia na Internet
Foley KM, Gelband H, editors. Improving palliative care for cancer [monograph on the Internet]. Washington: National Academy Press; 2001 [cited 2002 Jul 9]. Available from: http://www.nap.edu/books/0309074029/html/.

Homepage/Web site
Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/.

Parte de uma homepage/Web site
American Medical Association [homepage on the Internet]. Chicago: The Association; c1995-2002 [updated 2001 Aug 23; cited 2002 Aug 12]. AMA Office of Group Practice Liaison; [about 2 screens]. Available from: http://www.ama-assn.org/ama/pub/category/1736.html

BASE DE DADOS NA INTERNET

Acesso aberto:
Who's Certified [database on the Internet]. Evanston (IL): The American Board of Medical Specialists. c2000 - [cited 2001 Mar 8]. Available from: http://www.abms.org/newsearch.asp

Acesso fechado:
Jablonski S. Online Multiple Congenital Anomaly/Mental Retardation (MCA/MR) Syndromes [database on the Internet]. Bethesda (MD): National Library of Medicine (US). c1999 [updated 2001 Nov 20; cited 2002 Aug 12]. Available from: http://www.nlm.nih.gov/mesh/jablonski/syndrome_title.html

Parte de uma base de dados na Internet
MeSH Browser [database on the Internet]. Bethesda (MD): National Library of Medicine (US); 2002 - [cited 2003 Jun 10]. Meta-analysis; unique ID: D015201; [about 3 p.]. Available from: http://www.nlm.nih.gov/mesh/MBrowser.html Files updated weekly. Updated June 15, 2005

• **Ilustrações**: figuras e tabelas devem ser compreensíveis sem a necessidade de referência ao texto.

- Figuras: as fotografias devem ser bem nítidas, com alto contraste, ampliadas em preto e branco em papel brilhante, se apresentadas lâminas, as figuras devem ser numeradas consecutivamente em algarismos arábicos. As escalas devem ser indicadas por uma linha ou barra na figura, e referenciadas, se necessário, na legenda (por exemplo, bar = 1 mm etc.). Lâminas e gráficos devem ajustar-se tanto em uma coluna (8 cm) ou na largura completa (16.5 cm) da página, e devem ser menores que a página para permitir a inclusão da legenda. As letras e números nas figuras devem ter tamanho legível após a redução ou a impressão. Ilustrações coloridas somente podem ser aceitas se os autores assumirem os custos. Por outro lado, uma fotografia colorida ilustra a capa de cada fascículo de Memórias, e os autores são convidados a submeter para consideração da revista ilustrações com legendas de seus manuscritos que poderão vir a ilustrar a capa.

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