UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE – UFCSPA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Renata Oliveira Soares

Molecular Epidemiology and Virulence Gene Expression of *Enterococcus* spp. isolated from clinical samples in Southern Brazil

Porto Alegre
2018

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Tese apresentada como requisito parcial para a obtenção do título de Doutora, pelos Programas de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre – UFCSPA e em Ciências Farmacêuticas pela Universidade da Antuérpia – UA.

Orientador - Brasil: Prof. Dr. Pedro Alves d'Azevedo
Orientador - Bélgica: Prof. Dr. Paul Cos
Co-orientadora – Brasil: Prof. Dra. Juliana Caierão
Co-orientador – Bélgica: Prof. Dr. Guy Caljon

Porto Alegre
2018

Catalogação na Publicação

Oliveira Soares, Renata
Molecular Epidemiology and Virulence Gene Expression of Enterococcus spp. isolated from clinical samples in Southern Brazil / Renata Oliveira Soares. -- 2018.
128 f. : il., graf., tab. ; 30 cm.

Tese (doutorado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Ciências da Saúde, 2018.

Orientador(a): Pedro Alves d'Azevedo ; coorientador(a): Paul Cos, Juliana Caierão.

1. Enterococcus faecalis. 2. Enterococcus faecium. 3. Vancomycin resistance. 4. Virulence factors. 5. Biofilms. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

“Whatever the mind can conceive

and believe, it can achieve”.

(Napoleon Hill)

ACKNOWLEDGEMENTS
(AGRADECIMENTOS)

A Deus, em primeiro lugar, pela graça da vida, pela minha capacidade de sonhar e lutar por meus sonhos e por todas as pessoas maravilhosas que colocou em meu caminho.

À UFCSPA e às agências de fomento (CNPq e CAPES) que me deram oportunidade e suporte para a realização deste projeto.

Ao Prof. Dr. Pedro d'Azevedo, por me receber lá em 2012 como aluna de mestrado, por todas as oportunidades que me proporcionou, pelo conhecimento compartilhado e por ter sido apoio nos momentos em que precisei tomar decisões difíceis, sendo a mais recente, o ingresso na faculdade de Medicina. Obrigada professor!

Ao Prof. Dr. Paul Cos, pela oportunidade que tive como aluna de doutorado-sanduíche no Laboratório de Microbiologia, Parasitologia e Higiene da Universidade da Antuérpia. Além da chance de vivenciar a cultura de outro país e poder fazer pesquisa em um laboratório de ponta no exterior, também conheci pessoas incríveis que me ajudaram a tornar a dor da distância menos amarga.

À Prof. Dra. Juliana Caierão, pela torcida, amizade, conhecimento compartilhado e auxílio desde o mestrado. Você foi essencial nesta reta final Ju, obrigada por tudo!

Aos meus pais Mariza e Renato, sempre orgulhosos e amorosos. Obrigada pelos incentivos e por acreditarem em mim e nos sonhos que tenho. Amo vocês!

Ao meu parceiro e amor Gabriel, obrigada por ser meu porto-seguro, por me incentivar em tudo, torcer comigo, dar-me a mão para atingirmos juntos os meus sonhos. Por tornar esses sonhos seus também. Agradeço imensamente pelo apoio que me deu para que eu fizesse mestrado e depois doutorado, para que eu fosse estudar na Bélgica e agora para que eu cursasse Medicina. Você não existe e eu tenho a sorte de ter você em minha vida! Essa conquista é tua também.

À minha irmã Juliana e ao meu cunhado Bruno, por terem sempre torcido para que eu não esmorecesse frente aos obstáculos que surgiram, sendo base, apoio, auxílio, carinho e abraço. Obrigada por tudo que fizeram e fazem por mim!

Aos meus sogros Marisa e Dirnei, por serem minha segunda família e suporte em todos os momentos. Pela amizade, carinho, colo, incentivo e acima de tudo amor que me dão sem pedir nada em troca. Mil vezes, obrigada!

Aos amigos e colegas do Laboratório de Cocos Gram-positivos, aos que ficaram e aos que passaram pelo grupo ao longo desses sete anos. Agradeço por todos os momentos compartilhados, desde os ensinamentos na bancada e toda a ajuda experimental até os bolinhos de aniversário, o café da tarde e os abraços carinhosos aliviando angústias e aflições. Agradeço de coração a Adri, Gabi, Janira, Rebeca, Mari Mott, Kauana, Neidi, Bernard, Gustavo, Thiago, Fran, Dérik, Nathalia, Mari Sales, João, Thailan, Felipe, Carol, Andreia, Jussara, Fernanda, Cezar, Léo, Gui, Keli, Tiane, Aline, Vini e Janine. E aqueles que chegaram no finalzinho, mas deixaram seu carinho e contribuição, Rafael, Muri, Otávio, Lisi, Juliana, Maurício, Fernando e Roberto.

À Maíra, amiga de uma vida com quem pude compartilhar momentos especiais na Antuérpia, juntamente com a Luca e o Koen.

E por fim, agradeço aos meus queridos *Enterococcus* que me ensinaram a esperar, ter paciência, entender que nem tudo que parece negativo é necessariamente ruim e que quando perdemos a esperança é que a magia acontece. Com eles entendi que vivemos em um grande biofilme e que somos como nossos genes, expressamos emoções em diferentes condições. Hoje expresso meu carinho, admiração e gratidão a todos aqueles que despertaram em mim estes belos sentimentos, induzindo com altos níveis de amizade e suplementando com abraços, apoio e auxílio. Obrigada por fazerem parte da minha vida e da minha trajetória acadêmica.

ABSTRACT

Enterococcus faecalis and *Enterococcus faecium* are opportunistic pathogens, able to cause different types of infections, from ordinary urinary tract infections to life-threatening healthcare-associated ones. This capacity is directly associated with their ability to form biofilm and the presence of many virulence factors and resistance genes, giving them selective advantages in adverse conditions. Vancomycin-Resistant Enterococci (VRE) has become increasingly common in some settings around the world and their rapid and accurate identification, as well as the understanding of their clonal spread, can improve treatment and infection control policies. In this study, we evaluated the performance of a selective chromogenic medium (chromID™ VRE agar) using 184 clinical isolates of *Enterococcus* spp. (susceptible and resistant to vancomycin) and reference strains. Secondly, we characterized the susceptibility profile and clonal relationships of 115 VRE, recovered from inpatients attended at three general Hospitals of Porto Alegre, Brazil. Thirdly, we evaluated the ability to form biofilm of 123 *E. faecalis* clinical isolates in the presence and absence of 1% D-(+)-glucose and the expression of four biofilm-related genes (*ebpA, efaA, ace* and *gelE*) in eleven clinical isolates and *E. faecalis* ATCC 29212 by qPCR. Lastly, we evaluated the *E. faecalis* V583 biofilm formation capability in the absence and presence of human serum (1%, 5%, 25% and 50%) and the expression of five virulence genes *ebpA, efaA, ace, gelE* and *asa* in planktonic and sessile cells by qPCR. Although ChromID™ VRE agar had a very good sensitivity (95.52%), it presented a worrisome low specificity (30%). All VRE were identified as *Enterococcus faecium* and exhibited high-level resistance to vancomycin, resistance to teicoplanin, ampicillin, ciprofloxacin and 13.9% of them were resistant to high level of gentamicin. All VRE harbored *vanA* gene, 86.1% *esp* gene and 95.7% *acm* gene. PFGE profile analysis revealed a polyclonal

distribution with 23 clonal types encompassing 79 isolates, while 15 isolates exhibited unique patterns and 21 were non-typable. Considering biofilm formation, 1% glucose supplementation increased significantly the biofilm formation among *E. faecalis*. However, gene expression varied among them, showing that patterns of virulence gene expression may be dependent partially on the bacterial genetic background, rather than exclusively environmental conditions. On the other hand, it was observed an inhibition of the adhesion in the presence of 5% human serum compared to the control group. Planktonic cells of *E. faecalis* V583 exhibited upregulation of *asa* gene in 5%, 25% and 50% of human serum, while in the sessile cells, *efaA*, *gelE*, *ace* and *asa* genes were significantly upregulated in high concentrations of human serum (25% and 50%). A better understanding of this process as well as the application of these findings *in vivo* may help in the search for strategies to control the biofilm formation by this microorganism.

ABBREVIATIONS

ºC - degrees Celsius
% - percentage
µg - microgram
µL – microliter
µM - micromolar
h – hour
nm - nanometer
pmol - picomol
s – second

A

Ace – Collagen Adhesin
AMP - ampicillin
AS – Aggregation Substance
ATCC – American Type Culture Collection

B

BHI – Brain Heart Infusion
BLAST – Basic Alignment Search Tool
BSI – Bloodstream Infections

C

CAPES - Coordination for the Improvement of Higher Level -or Education- Personnel
CAUTI – Catheter-associated urinary tract infection
CDC – Centers for Disease Control and Prevention
cDNA- Complementary DNA
CIP - ciprofloxacin
CLSI – Clinical and Laboratory Standards Institute
CNPq - National Council for Scientific and Technological Development
CT – clonal type

D

DNA – deoxyribonucleic acid

E

Esp – Enterococcal Protein Surface

F

FAPERGS - Foundation of Research Support of the State of Rio Grande do Sul

G

GI – Gastrointestinal tract

H
HAI – Healthcare Associated Infection
HA –Hospital A
HB –Hospital B
HC –Hospital C
HLG – High Level of Gentamicin
HS – Human Serum

I
IE – Infective Endocarditis
ICU – Intensive Care Unit

M
MIC – Minimium Inibitory Concentration
MBC – Minimum Bactericidal Concentration
MSCRAMMS - Microbial Surface Components Recognizing Adhesive Matrix Molecules

P
PBP – Penicillin-Binding Protein
PCR – Polymerase Chain Reaction
PFGE – Pulsed Field Gel Electrophoresis

Q
qPCR - quantitative Polymerase Chain Reaction

R
RNA – Ribonucleic Acid

T
TBE - Tris/Borate/EDTA
TEI - teicoplanin
TSB – Tryptic Soy Broth
TSA – Tryptic Soy Agar
TSBg - Tryptic Soy Broth plus Glucose

V
VAN - vancomycin
VRE – Vancomycin-Resistant Enterococci

U
UFCSPA - Federal University of Health Sciences of Porto Alegre
UTI - Urinary Tract Infection

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used as control. A. Expression of planktonic cells of *E. faecalis* V583. B. Expression of sessile cells of *E. faecalis* V583. Error bars represent standard deviation. Statically differences obtained when compared with control ($* P < 0.05$).

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CHAPTER 1. INTRODUCTION

1.1 GENUS *Enterococcus*

Genus *Enterococcus* includes Gram-positive bacteria, non-spore forming, facultative anaerobes, arranged in pairs or short chains whose shape is spherical or ovoid. These bacteria can grow in high salt concentrations and in a range of temperatures (10-45ºC). The ability to hydrolyse esculin in the presence of 40% of bile salts and the absence of enzyme are also typical characteristics of this genus (LEBRETON et al., 2014).

Fifty-eight species of *Enterococcus* were recognized between 1992 and 2017 (EUZÉBY, 2018). Due to their ability to survive under adverse conditions, they can be found in a variety of environments such as soil, water, plants as wells as and colonizing the oral cavity, genitourinary and gastrointestinal (GI) tract of humans and animals (BYAPPANAHALLI et al., 2012; HIGUITA & HUYCKE, 2014).

Enterococcus faecalis and *Enterococcus faecium* are the main species associated whit human infections, being responsible for the majority of reported cases (ARIAS & MURRAY, 2012). Meantime, although in a small proportion, other enterococcal species including *E. gallinarum, E. casseliflavus, E.hirae, E. raffinosus*, *E. avium, E. mundtii* and *E. durans* have also been reported in human infections. In some scenarios, however, these species may be underestimated due to the frequent misidentification (PRAKASH et al., 2005; ESCRIBANO et al., 2013; HIGUITA & HUYCKE, 2014; BOURAFA et al., 2015; SHARIFI-RAD et al., 2016).

1.1.1 *E. faecalis* and *E. faecium*

Infections caused by *E. faecalis* and *E. faecium* are considered a global problem, once it can be difficult to manage and treat (HIGUITA & HUYCKE, 2014; KRISTICH et al., 2014). It is known that *E. faecium* is intrinsically more resistant to antibiotics than *E. faecalis*. This species has emerged as one of the main multidrug-resistant pathogens in the hospital environment due to its intrinsical resistance characteristics plus its genomic plasticity, leading to the acquisition and dissemination of mobile genetic elements carrying resistance genes (GAO et al., 2018).In this context, *E. faecium* expressing resistance to vancomycin, ampicillin, and high-levels of aminoglycosides is the major phenotype among clinical isolates, impairing therapeutical options (PALMER et al., 2011; LEBRETON et al., 2013; HIGUITA & HUYCKE, 2014; RAZA et al., 2018).

These well-adapted *E. faecium* lineages designs a clonal dissemination pattern, where most strains of *E. faecium* causing nosocomial infections are part of the same sub-population (CATTOIR & LECLERCQ, 2013; PRIETO et al., 2016).

Despite *E. faecium* importance (mainly because of their resistance patterns), *E. faecalis* is responsible for the largest number of cases of enterococcal infection, being 10 times more prevalent than other species of enterococci (ARIAS & MURRAY, 2012; SHOKOOHIZADEH et al., 2013). It is suggested that they are widely adapted to the host due to their commensal relationship. Besides, they also possess virulence characteristics, such as the ability to form biofilm, which facilitate the processes of colonization and opportunistic infection (SHEPARD & GILMORE, 2002; SURIYANARAYANAN et al., 2018).

1.2 ENTEROCOCCAL DISEASE

Usually, enterococci coexist with the host. However, different factors, such as broad-spectrum antimicrobials and host immunosuppression, may alter the role of this microorganism, from commensal to opportunistic pathogen, breaking the symbiotic relationship (SHEPARD & GILMORE, 2002; LITTMAN & PAMER, 2011).

Enterococci are able to cause different types of infections. The urinary tract is the most common site of enterococcal infection, i.e. cystitis, prostatitis and epididymitis in older man and uncomplicated cystitis in young women. Intra-abdominal, pelvic, and soft tissue are also sites of enterococcal infections, but rarely presented as monomicrobial infection (ORSI & CIORBA, 2013; HIGUITA & HUYCKE, 2014).

Enterococci is the third cause of bloodstream infections (BSI) and infective endocarditis (IE), which are the most serious and often life-threatening infections caused by this microorganism (BEGANOVIC et al., 2018; PERICÁS et al., 2015). Rarely, they cause osteomyelitis, septic arthritis, and pneumonia (KOW & FERZANDI, 2013; HIGUITA & HUYCKE, 2014) as well as diseases involving central nervous system (WANG et al., 2014; PATEL et al., 2016).

Indeed, enterococci are among the main aetiological agents of healthcare-associated infections (HAI) (HIDRON et al., 2008; SIEVERT et al., 2013). In the 1970s and 1980s, the occurrence of antimicrobial resistance led to the emergence of this genus, being a subject of major concern among infection control staff (ARIAS & MURRAY, 2012). Between 2011 and 2014, 365,490 cases of HAIs in the United States were reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention. *E. faecalis, E. faecium* and other species of enterococci were

responsible for 7.4% (30,034), 3.7% (14,942) and 3.6% (14,694) of the cases of nosocomial infections, respectively (WEINER et al., 2016).

Because of its opportunistic nature, the risk factors for enterococcal infection are mainly advanced age, being female, long-term hospitalization, organ transplantation, presence of medical devices, multiple antimicrobial therapy and immunosuppression (CONWAY et al., 2016; MONTESERIN & LARSON, 2016; MEDINA-POLO et al., 2017; POULADFAR et al., 2017).

1.2.1 Treatment and antimicrobial infection

Treatment of enterococcal infections could be challenging and it depends on several factors such as site and nature (monomicrobial or polymicrobial) of infection, as well as and susceptibility to β-lactams, aminoglycosides, and glycopeptides or to the combination of these antimicrobial classes (HIGUITA & HUYCKE, 2014).

For systemic infections, it is recommended the combination of high doses of gentamicin with penicillin or ampicillin. For ampicillin-resistant isolates, glycopeptides (vancomycin or teicoplanin) in association with gentamycin are the alternative therapy, although eradication rates are lower. On the other hand, the treatment of vancomycin-resistant enterococci (VRE) will depend on the antibiogram and the *Enterococcus* species. Linezolid or daptomycin may be used to treat infections by *E. faecalis* and *E. faecium* resistant to vancomycin, however, the combination of quinupristin/dalfopristin may be administered only in case of *E. faecium* infections once *E. faecalis* is intrinsically resistant to this drug (SHEPARD & GILMORE, 2002; ARIAS & MURRAY, 2012).

As mentioned before, the main reason for the recognition of enterococci as an important nosocomial pathogen is their resistance to different antimicrobials, which

gives them selective advantages and contributes to their adaptation in adverse environments, colonization of the GI tract and spread among patients. Such resistance may arise from mutations in the nucleic acid sequence or be acquired by horizontal gene transfer. In general, it involves transformation and acquisition of genetic determinants of resistance, in plasmids and transposons, from another microorganism carrying the phenotype (GILMORE et al., 2013; KRISTICH et al., 2014).

Enterococci are intrinsically resistant to cephalosporins, sulfonamides, clindamycin and low levels of β-lactam and aminoglycosides, due to chromosome-mediated genes. Acquired resistance may arise to virtually to all commonly used antimicrobial agents classes: macrolides, chloramphenicol, tetracycline, fluoroquinolones, and glycopeptides, as well as high levels of resistance to aminoglycosides and β-lactams (Figure 1) (SHEPARD & GILMORE, 2002; ARIAS & MURRAY, 2012; KRISTICH et al., 2014).

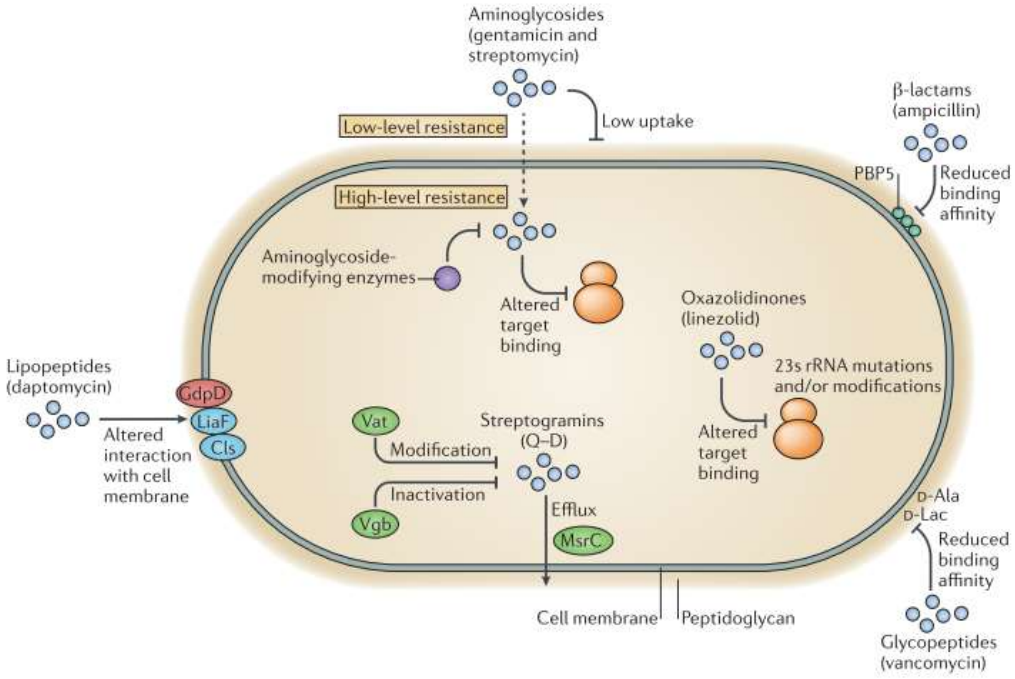


Fig 1. Mechanism of enterococcal antimicrobial resistance (Retrieved from ARIAS; MURRAY, 2012).

Ampicillin belongs to the β-lactams antibiotics, whose inhibit the peptidoglycan synthesis by binding to the penicillin-binding proteins (PBPs). Resistance to β-lactams is associated with the production of a low-affinity PBP or, less frequently, the production of β-lactamases. While ampicillin resistance in *E. faecalis* is rare, it occurs in 90% of *E. faecium* and is associated with PBP5 production, a constitutive low-affinity enterococcal PBP (ARIAS & MURRAY, 2012; CATTOIR & GIARD, 2014).

Gentamicin is an aminoglycoside, which acts by binding the bacterial 30S small ribosomal subunit, inhibiting the protein synthesis. As enterococci are intrinsically resistant to low levels of aminoglycoside, the treatment includes an association of aminoglycoside (high-level) with β-lactams or glycopeptides, but the resistance to high levels of aminoglycosides impairs the synergism of treatment. This resistance occurs due to the production of aminoglycoside-modifying enzymes, which lead to loss of synergism and decrease the bactericidal effect (SHEPARD; GILMORE, 2002; ARIAS; MURRAY, 2012).

Ciprofloxacin is a fluoroquinolone that acts inhibiting cell division, commonly used to treat urinary tract infections. Resistance to this antimicrobial occurs by mutations in *gyrA* and *parC* genes that encode, respectively, DNA gyrase and Topoisomerase IV (LI et al., 2015).

Vancomycin and teicoplanin are glycopeptides, whose mechanism of action involves binding to the terminal D-alanine-D-alanine dipeptide of bacterial cell wall inhibiting the synthesis of peptidoglycan. Resistance to glycopeptides is due to the modification in the ending binding target D-alanine by D-lactate (high levels of resistance) or D-serine (low levels of resistance) (CATTOIR & LECLERCQ, 2013; FARON et al., 2016). So far, nine genes encoding glycopeptide resistance have been reported (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*) (Table 1).

vanA and *vanB* are the most prevalent in HAI and highly transferable to other pathogens. VanA phenotype is characterized by high levels of vancomcyin and teicoplanin resistance, while and VanB determines various levels of inducible resistance to vancomcyin and susceptibility to teicoplanin. (DUTKA-MALEN et al., 1995; PERICHON et al., 1997; FINES et al., 1999; MCKESSAR et al., 2000; LEBRETON et al., 2011; BOYD et al., 2008; XU et al., 2010). Recently, it was first described in Europe an *E. faecium* isolated, from a patient with bacteremia, harboring both *vanA* and *vanB* genes (PAPAGIANNITSIS et al., 2017).

Table 1. Summary of the Phenotypic and Genotypic Characteristics of the Alphabet VREs Operons (Retrieved from Ahmed & Baptiste, 2017)

Van-operon	*Common carrier spp.*	*Degree level of resistance vancomycin vs. teicoplanin*	*Phenotypic expressions*	*Location & mobility*
vanA	*E. faecium* *E. faecalis*	High for both	Inducible	Chromosome Transferable
vanB; vanB1, B2, B3	*E. faecalis* *E. faecium*	High-variable to vancomycin Susceptible to teicoplanin	Inducible	Chromosome Transferable
vanC; vanC1, C2, C3, C4	*E. gallinarum* *E. casseliflavus* *E. flavescens*	Low to vancomycin Susceptible to teicoplanin	Constitutive Inducible 	Chromosome —
vanD; vanD1, D2, D3, D4, D5	*E. faecium*	Low to high for both	Constitutive Inducible	Chromosome —
vanE	*E. faecalis*	Low-moderate to vancomycin Susceptible to teicoplanin	Inducible	Chromosome —
vanG; vanG1, G2	*E. faecalis*	Low to vancomycin Susceptible to teicoplanin	Inducible	Chromosome Transferable
vanL	*E. faecalis*	Low to vancomycin Susceptible to teicoplanin	Inducible	Chromosome —
vanM	*E. faecium*	High for both	Inducible	Unknown Transferable
vanN	*E. faecium*	Low to vancomycin Susceptible to teicoplanin	Constitutive	Plasmid Transferable

VRE, vancomycin resistance enterococci.

Linezolid is an oxazolidinone antibiotic that inhibits proteins synthesis, commonly used to treat infections caused by gram-positive bacteria, such as community-acquired and nosocomial pneumonia, skin and soft tissue infections and VRE infections resistant to penicillins. This antimicrobial has no activity against gram-negative bacteria. Resistance to linezolid is associated with mutations in the *23S rRNA*

gene, and it is considered an emerging problem (AGER & GOULD, 2012; KRISTICH et al., 2014; BI et al., 2018).

Quinupristin/dalfopristin is a combination of two streptogramins (A and B) that inhibit protein synthesis after their interaction with the 50S ribosomal subunit. This antimicrobial is active against *E. faecium*, while *E. faecalis* is intrinsically resistant to the drug. Acquired resistance among *E. faecium* is commonly associated with streptogramin A resistance genes and can occur by enzymatic modification of the antibiotic, is commonly associated with the presence of *vatD* and *vatE*, which encode acetyltransferases that inactivate the antibiotic (ARIAS & MURRAY, 2012; KRISTICH et al., 2014).

Daptomycin is a lipopeptide antibiotic, derived from *Streptomyces roseosporus* fermentation, which is active only against gram-positive bacteria. Its mechanism of action involves binding to bacterial membranes, in the presence of calcium ions, causing depolarization and inducing the rapid inhibition of protein, DNA, and RNA synthesis, resulting in bacterial cell death (STEENBERGEN et al., 2005). In enterococci, daptomycin resistance is associated with alterations in genes responsible for regulatory systems of cell-envelope homeostasis and stress-response, as well as genes responsible for producing enzymes involved in the metabolism of phospholipids (TRAN et al., 2015).

1.2.2 Vancomycin Resistant Enterococci

In most regions, the majority of enterococci causing HAI are resistant to vancomycin, therefore the treatment is a challenge, once these bacteria are also resistant to the other antimicrobials most commonly used to treat these infections

(HIGUITA & HUYCKE, 2014). There is evidence that VRE may act as reservoir and sources of other antimicrobial-resistant genes (AHMED & BAPTISTE, 2017).

VRE infection starts with the colonisation of the host GI tract, most commonly in critically ill patients. After the establishment as a member of the GI microbiota, they can take advantage of any disruption of the intestinal flora and quickly proliferate in the gut (MILLER et al., 2016). Previous treatment with vancomycin is a risk factor for the gut colonization with VRE. Besides, the risk to develop VRE infection is 9 times higher in hospitalized patients colonized than in non-colonized, especially among children (FLOKAS et al., 2017).

Other risk factors for the development of VRE infection includes physical proximity with infected or colonized patients, long-term hospitalization, multiple antimicrobial therapy, patients undergoing transplantation, comorbidities such as diabetes and renal failure, and presence of medical devices (ARIAS & MURRAY, 2012; SHORMAN & AL-TAWFIQ, 2013; ADESIDA et al., 2017). The risk of death by VRE infection is higher compared to VSE infection, once the therapeutic options are limited in the first case. For example, DA SILVA et al., (2014) reported a risk of death by VRE bacteremia 2.73-fold higher than by VSE bacteria.

It is known that enterococci can survive for up to 4 months on surfaces and persist for up to 60 min on the skin. In the hospital environment, hands of healthcare professionals, if improperly sanitized, may serve as a vector for the VRE spread between patients (FARON et al., 2016). Controlling the spread of these resistant bacteria are sorely needed. Hence, the implementation of a strong infection control program in the healthcare facilities, as well as, specific policies related to the bacterial prevalence and rates of resistance, may minimize the risk of transmission and outbreaks (HIGUITA & HUYCKE, 2014).

Clinical use of vancomycin started in the 1960s and the first report of VRE occurred in England in 1988 (UTTLEY et al., 1988). After that, VRE has been quickly spread among hospitals, people and animals, countries and continents (WILLEMS et al., 2005; ARIAS & MURRAY, 2012; CATTOIR & LECLERCQ, 2013).

In Brazil, the first case was described in 1996, in a hospital of Curitiba, Southern Brazil. Besides vancomycin resistance, the strain exhibited resistance to teicoplanin, ampicillin, and to high levels of gentamicin and streptomycin (DALLA COSTA et al., 1999). In 1997, a case of *E. faecium* carrying *vanA* was described in a patient with meningitis in São Paulo, Southeast Brazil (ZANELLA et al., 1999). In 2000, it was reported the first case of vancomycin resistant *E. faecalis* (VRE_{fl}) isolated from an ICU patient, in Porto Alegre (D'AZEVEDO et al., 2000). However the epidemiology changed, and in 2014, in Porto Alegre, RESENDE et al., (2014) described a clonal outbreak of vancomycin resistant *E. faecium* (VRE_{fm}) carrying the *vanA* gene.

During 2015, the SENTRY Antimicrobial Surveillance Program evaluated 8,072 gram-positive isolates collected from 69 medical centers in the U.S. Census Divisions and among the *Enterococcus* sp. isolates, 71.1% were VRE (PFALLER et al., 2017).

VEGA & DOWZICKY (2017) reinforce the importance of VRE in Brazil. They described the antimicrobial susceptibility profile of gram-positive and gram-negative bacteria collected between 2004 and 2015 in nine countries of Latin America, including Brazil. They observed 40.8% of VRE_{fm} among all countries, being Brazil the country with the highest rate of VRE (77.3%).

As a consequence of the increasing of VRE isolation in many regions around the world, studies have been performed focusing on the epidemiological characterization of these isolates in order to understand the VRE spread. Different tools have been used to obtain the genetic relatedness of the strains, such as Pulsed

Field Gel Electrophoresis (PGFE) and Multilocus Sequence Typing (MSLT), being the MLST the only methodology able to compare the data obtained from different laboratories (PRIETO et al., 2016).

The clonal complex 17 (CC17), identified by MLST, is a lineage of *E. faecium*, well-adapted to the hospital environment and responsible to the major of VRE_{fm} infections worldwide (TOP et al., 2008b). This lineage has been reported in epidemiological studies performed in different countries that evaluated the genetic relatedness by MLST, such as Iran, China, USA, Brazil (PALAZZO et al., 2011; DA SILVA et al., 2012; CORREIA et al., 2014; YANG et al., 2015; ALVES et al., 2017; MICHAEL et al., 2017; SACRAMENTO et al., 2017). In contrast, *E. faecalis* are less associated with one dominant clone. Despite that, CC2, CC40, and CC87 are the most common lineages of *E. faecalis* present in hospital-associated infections (MIKALSEN et al., 2015; PRIETO et al., 2016).

In Brazil, most of the epidemiological studies were performed with VRE isolates from São Paulo. In 2008, D'AZEVEDO et al., (2008) evaluated the genetic relatedness by PFGE of 37 VREs (20 VRE_{fm} and 17 VRE_{fl}) isolated in a teaching hospital of São Paulo with the first VRE isolated in Brazil, and they observed seven distinct clonal types and all different from the first VRE. In 2011, PALAZZO et al., (2011) characterized by PFGE and MLST 22 VRE_{fm} isolated from different patients during an outbreak in a University Hospital in Southeast Brazil, and they identified new sequences types but all belonged to the CC17. The same was observed by DA SILVA et. al., (2012) that identified 53 VRE_{fm} isolated from two hospitals in Ribeirão Preto, São Paulo. All grouped in 9 STs, being 8 STs belonging to CC17 and one from a different clonal complex (ST658).

More recently, a study evaluated 2633 VRE from 26 hospitals, isolated during an 18-year period. PFGE was performed to 153 VRE_{fl} and 125 VRE_{fm} randomly selected and grouped both species in four clusters. The PFGE clusters were also evaluated by MLST, and VRE_{fl} isolates were identified as belonging to CC2, CC4 and CC9 and all VRE_{fm} belonging to CC17 (SACRAMENTO et al., 2017).

1.3 VIRULENCE FACTORS OF ENTEROCOCCI

Enterococci possess many virulence factors which give them pathogenic potential to break the stable relationship whit the host and become a pathogen in the human body, as well as, a selective advantage to survive under adverse conditions. Changes in the ecology and different virulence determinants have been associated with enterococcal infection. However, this virulence is complex and multifactorial without a major molecule or event (BALLERING et al., 2009; GILMORE et al., 2013).

As previously mentioned, *E. faecalis* is generally more virulent than *E. faecium* However, with the advent of sequencing, new insights have reinforced the virulence potential of *E. faecium* (SOHEILI et al., 2014; AL-TALIB et al., 2015; GAO et al., 2018; JAHANSEPAS et al., 2018).

1.3.1 Virulence genes

Most virulence factors among enterococci are related to adhesion in biotic and abiotic surfaces. Enterococcal surface protein (Esp) is a cell wall-associated protein, coded by a gene located on a pathogenicity island, that contributes to the persistence of *Enterococcus* at the site of infection and can modulate inflammation. Firstly

described in *E. faecalis*, *esp* gene has a homologue which is also highly disseminated among *E. facium* (*esp*$_{fm}$) (SHANKAR et al., 1999; EATON & GASSON, 2002; SAVA et al., 2010; ZOU & SHANKAR, 2016; GAO et al., 2018). Esp increases the *E. faecalis* and *E. faecium* ability to form biofilm and contributes to colonization of heart valves (TENDOLKAR et al., 2004; HEIKENS et al., 2007; HEIKENS et al., 2011).

Collagen Adhesin (Ace) is a protein that belongs to the MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules), which mediates adherence to collagen and laminin and contributes to the ability of *Enterococcus* sp. to colonize aortic valves and cause endocarditis. It is encoded by *ace* gene in *E. faecalis* and *acm* gene in *E. faecium* (NALLAPAREDDY & MURRAY, 2006; NALLAPAREDDY et al., 2008; SINGH et al., 2010).

Gelatinase (GelE) is a secreted factor, regulated by the Fsr quorum sensing in *E. faecalis.* This protease is able to hydrolyse gelatin, casein, collagen, hemoglobin and is the main mediator of pathogenesis in endocarditis. Its enzymatic activity is required in the first steps of the biofilm formation, as well as its development (HANCOCK & PEREGO, 2004; THURLOW et al., 2010; GARSIN et al., 2014).

Enterococci change plasmids very easily mainly because of aggregation substance (AS) which is a group of surface proteins, encoded by pheromone-responsive gene, that mediates the transfer of plasmids favoring bacterial aggregation. Its adherence properties also enhance *E. faecalis* virulence by accelerating the biofilm formation (CHUANG-SMITH et al., 2010; GARSIN et al., 2014).

Endocarditis and biofilm-associated pili (*ebp* locus) is a pilin gene cluster of *E. faecalis* and *E. faecium* that comprises three structural subunits EbpA, EbpB and EbpC and a pilus-specific sortase. *ebp* pili encodes surface proteins, contributes to the colonization, and infection of host tissues, has a role in the initial adherence to

platelets, fibrinogen and collagen and affect the biofilm biogenesis (NALLAPAREDDY et al., 2006; NIELSEN et al., 2013; SILLANPÄÄ et al., 2013).

Finally, *E. faecalis* antigen A (EfaA) is an adhesin, considered the major surface antigen of *E. faecalis.* It has been associated with infective endocarditis and also found in therapy-resistant endodontic infections (LOWE et al., 1995; PREETHEE; KANDASWAMY & HANNAH, 2012). It was recently reported an influence of the *efaA* in the biofilm formation induced by the antibiotic (KAFIL et al., 2016).

1.3.2 Biofilm

Enterococci are strongly associated with infections related to medical devices once they are capable to attach firmly to the surface of material and form biofilms, as a way to resisting shear forces. Biofilms are populations of microbial cells attached at a biotic or abiotic surface, typically surrounded by a self-produced matrix of extracellular polymeric substances (EPS). Studies have demonstrated that bacteria use their ability to form biofilm in the colonization and infection processes, as a survival strategy in hostile environments. It is believed that the most part of the microbial existence in Earth is within a biofilm community (MONDS & O'TOOLE, 2009; GARSIN & WILLEMS, 2010; GUPTA et al., 2015).

Biofilm formation is dependent on different factors, such as pH, temperature, CO_2, nutrients, surface type and cell density (PILLAI et al., 2004). Presence of supplements as carbohydrates and serum are examples of important factors that influence the enterococci adhesion at surfaces, as well as the metabolism of biofilm production of this bacteria (MOHAMED & HUANG, 2007).

The process of biofilm formation is complex and involves different stages: attachment and immobilization on a surface by physical forces, cell-to-cell interaction,

accumulation, maturation, secretion of the EPS, dispersal and return to the planktonic form. Initially, the attachment step is reversible and starts with the adhesion of planktonic cells on a surface. After that, this adhesion becomes irreversible, the microbial cells start to grow, and form a three-dimensional biofilm structure (Figure 2) (GUPTA et al., 2015; MONDS & O'TOOLE, 2009).

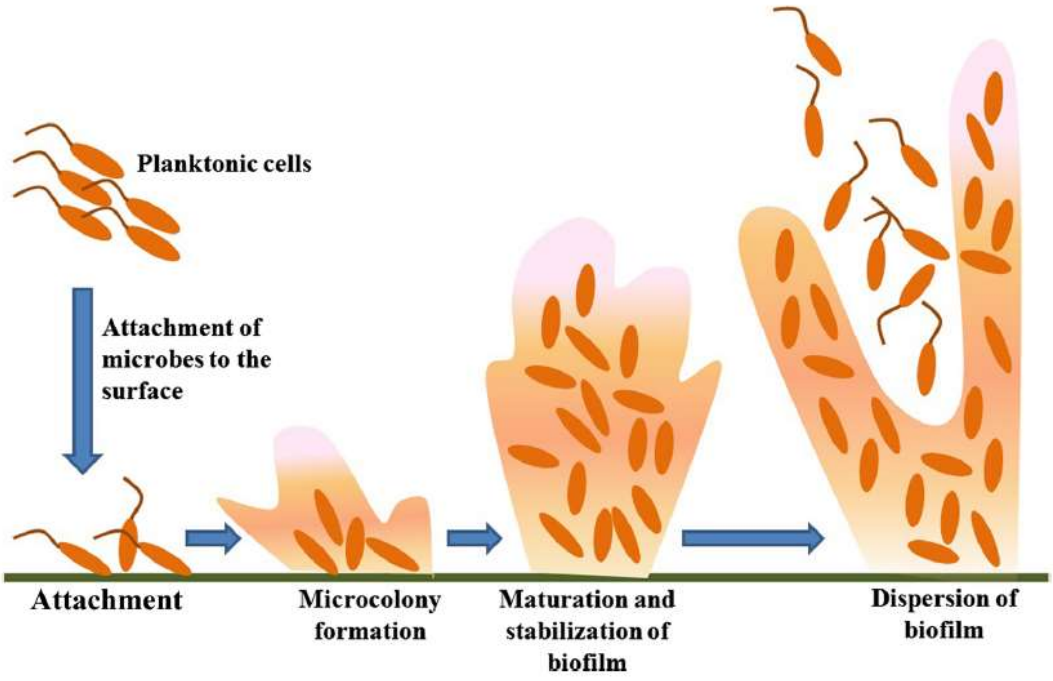


Fig.2 Stages of biofilm formation (Retrieved from GUPTA et al., 2015)

Several genetic determinants have been reported in the literature as contributing to form the biofilm, predominantly in *E. faecalis,* and the number of genetic factors required to the biofilm formation has increased, reinforcing the complexity of these communities. Among these biofilm-related genes, there are surface proteins located in the genome or plasmids, enzymes as sortase, autolysin and proteases, regulatory genes and genes of the enterococci metabolism (Figure 3) (MOHAMED & HUANG, 2007; PAGANELLI et al., 2012).

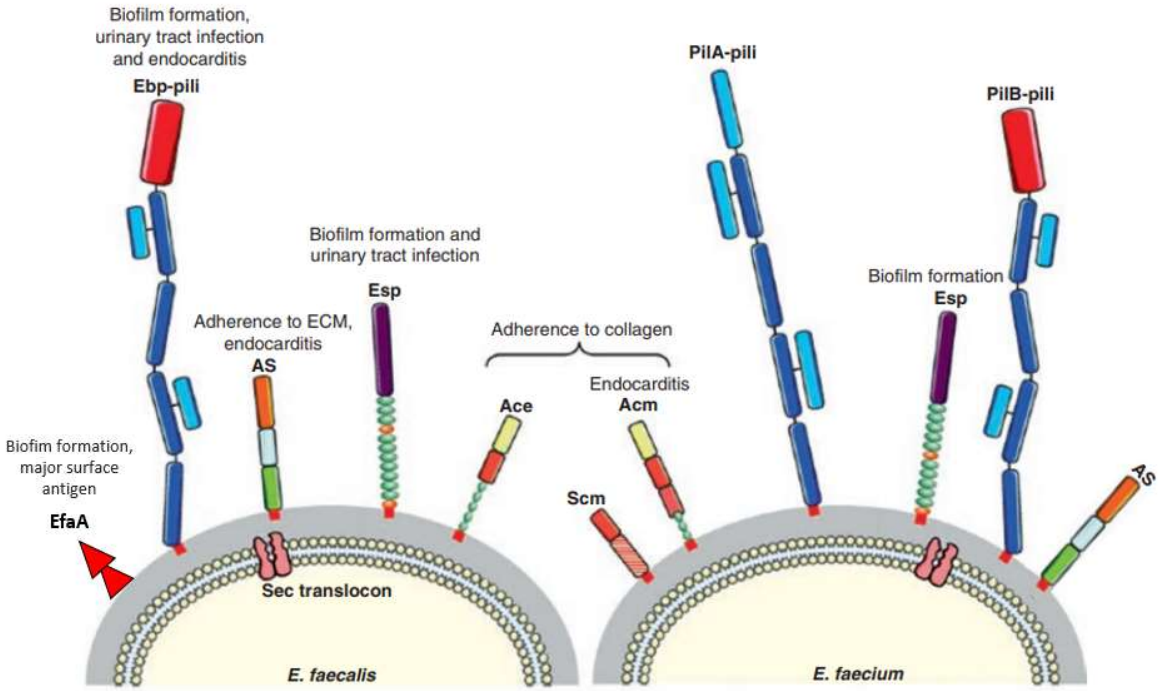


Fig. 3 Schematic representation of biofilm-associated proteins and structures of *E. faecalis* and *E. faecium*. (Retrieved and adapted from HENDRICKX et al, 2009).

Several types of chronic infections are strongly associated with biofilm formation, making these infections difficult to treat and eradicate (Figure 4). It is a mode of bacteria to survive in adverse environments, protection against stress conditions, phagocytosis, dehydration and antimicrobial therapy and also can increase the bacterial pathogenicity (MOHAMED & HUANG, 2007; GUPTA et al., 2015).

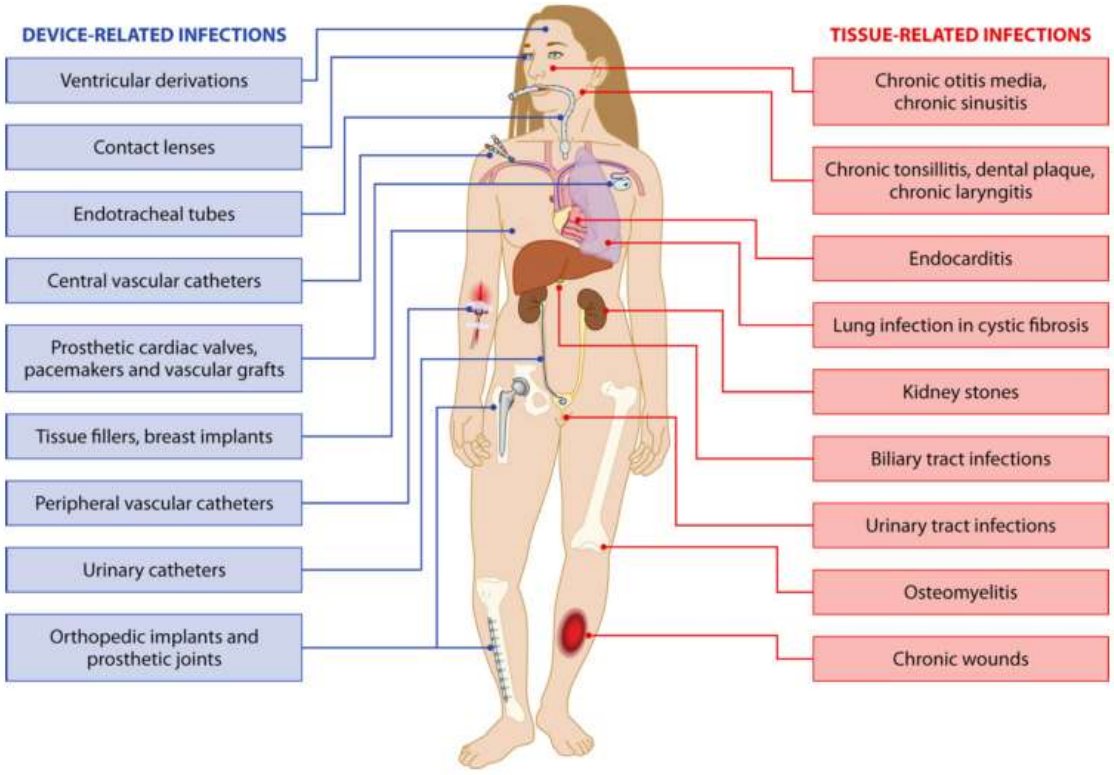


Fig. 4 Biofilm-related infections (Retrieved from LEBEAUX; GHIGO; BELOIN, 2014).

LEBEAUX et al., (2014), described some strategies that can avoid the biofilm formation: reduction in the number of implanted devices, early removal of unnecessary devices, systemic antibiotic prophylaxis during device implantation, antibiotic coating of implanted devices and antibiotic lock therapy. Indeed, different lock therapy regimens have been tested against *E. faecalis* biofilm, however, in some cases, the biofilm was not completed eradicate by the tested concentrations (LUTHER et al., 2016, 2017; ALONSO et al., 2018).

Since a biofilm is established, the treatment of the infection is a challenge and involves, in most cases, the removal of the indwelling devices, or chronic biofilm suppressive treatment with antibiotic combination therapy (WU et al., 2015). In biofilms, the Minimum Bactericidal Concentration (MBC) required for sessile cells can be approximately 10-1000 times higher than the MBC for planktonic cells, impacting

the treatment once the effective antimicrobial therapy is impossible to administrate due to toxicity (MOHAMED & HUANG, 2007; WU et al., 2015).

Bacteria can naturally exhibit resistance or tolerance in the presence of the antimicrobial. Resistance is often due to genetic alterations and occurs by numerous mechanisms, such as efflux pump, reduced permeability to antibiotics, enzymatic activity and modification of the antibiotic target. On the other hand, the antimicrobial tolerance can be genetic, when there are genes associated with the reduced affinity by the antibiotic, or phenotypic when the environment affects the antibiotic action. Biofilm is a mixture of resistance and tolerance, resulting in decreased antibiotic penetration, slow growth rate, altered metabolism and presence of persister cells (Figure 5) (ABDALLAH et al., 2014; KESTER; FORTUNE, 2014; LEBEAUX et al., 2014; OLSEN, 2015).

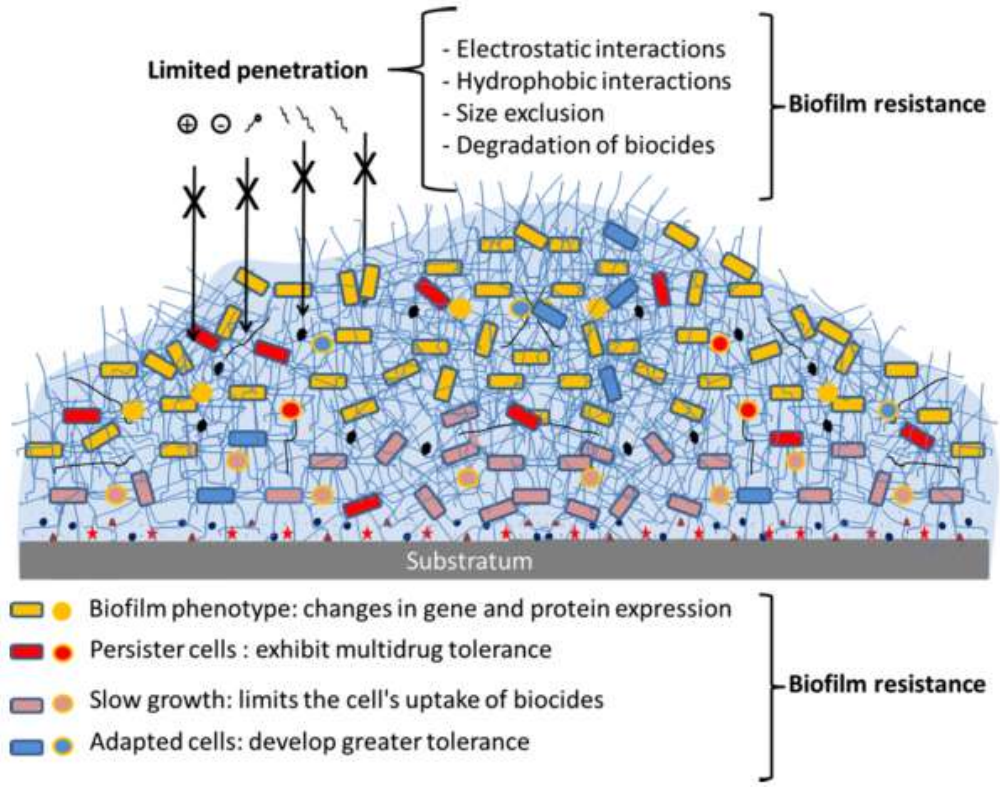


Fig. 5 Mechanisms of biofilm resistance to antimicrobial agents (Retrieved from ABDALLAH et al., 2014).

CHAPTER 2. AIMS OF THE STUDY

2.1 GENERAL AIM

The general aim of this research was to characterize, epidemiologically and genotypically isolates of *Enterococcus* spp. from the Gram-Positive Laboratory Microrganism Bank (Federal University of Health Sciences of Porto Alegre, Brazil). The isolates were recovered from clinical samples of patients attended in hospitals of Porto Alegre, Brazil.

2.2 SPECIFIC AIMS

- To evaluate the performance of a commercial agar to detect VRE.
- To evaluate the susceptibility profile of VRE isolates.
- To detect the presence of vancomycin resistant genes in Vancomycin Resistant Enterococi by conventional PCR.
- To determine clonal relationship among VRE isolates by PFGE.
- To detect the presence of virulence factors in *E. faecalis* and *E. faecium* by conventional PCR.
- To Analyse the influence of environmental factors, such as presence and absence of 1% glucose and presence of different concentrations of human serum (1%, 5%, 25% and 50%) in biofilm formation of *E. faecalis*
- To quantify the expression of virulence genes associated with biofilm in different environmental conditions.

CHAPTER 3. METHODOLOGY

Materials and specific methods performed in this research are described in the respective manuscripts, presented in CHAPTER 4. RESULTS.

The present research was developed in the Laboratory of Cocos Gram Positives (LCGP) of the Federal University of Health Sciences of Porto Alegre, Brazil. The Ph.D. scholarship was funded by the Coordination for the Improvement of Higher Education Personnel (CAPES).

Part of this research was developed in the Laboratory for Microbiology, Parasitology and Hygiene (LMPH) of the University of Antwerp, Belgium. The Sandwich Ph.D. scholarship was funded by the National Council for Technological and Scientific Development (CNPq) through the Science without Borders Program (Process number: 201982/2015-3).

CHAPTER 4. RESULTS

The results of this study are presented in the format of a published article or manuscript formatted according to the journal in which it will be submitted.

4.1 EVALUATION OF A SELECTIVE CHROMOGENIC MEDIUM FOR DETECTING VANCOMYCIN-RESISTANT ENTEROCOCCI

Soares RO, Rossato AM, Sambrano GE, Tolfo NCC, Caierão J, Paim TGDS, d'Azevedo PA. Evaluation of a selective chromogenic medium for detecting vancomycin-resistant enterococci. Braz J Microbiol. 2017 Oct - Dec; 48(4):782-784. Doi: 10.1016/j.bjm.2017.03.005.

BRAZILIAN JOURNAL OF MICROBIOLOGY 48 (2017) 782–784

BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Clinical Microbiology

Evaluation of a selective chromogenic medium for detecting vancomycin-resistant enterococci

Renata Oliveira Soares, Adriana Medianeira Rossato, Gustavo Enck Sambrano, Neidimar Cezar Corrêa Tolfo, Juliana Caierão, Thiago Galvão da Silva Paim, Pedro Alves d'Azevedo*

Federal University of Health Sciences of Porto Alegre, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 7 June 2016
Accepted 1 March 2017
Available online 8 June 2017

Associate Editor: Agnes Figueiredo

ABSTRACT

Rapid identification of vancomycin-resistant enterococci (VRE) can assist in choosing the appropriate treatment and preventing VRE spread. The performance of chromID™ VRE agar was evaluated using 184 clinical isolates of *Enterococcus* spp. and reference strains. The test had a sensitivity of 95.52% but a low specificity of 30%.

Vancomycin-resistant enterococci (VRE) are among the major agents of healthcare-associated infections and are considered a public health problem. Rapid VRE identification can assist in choosing the appropriate treatment and preventing VRE spread.[1,2] The aim of this study was to evaluate the performance of a selective chromogenic medium for the detection and differentiation of vancomycin-susceptible and -resistant *Enterococcus faecium* and *Enterococcus faecalis*.

Vancomycin-susceptible enterococci (VSE) isolated from cases of infection ($n = 50$) and VRE clinical isolates ($n = 134$), including those collected from surveillance rectal swab cultures ($n = 62$) and from cases of infection ($n = 72$), were evaluated (Table 1). The following reference strains were also included: VRE strain (*E. faecium*, $n = 1$) and VSE strains (*E. hirae*, $n = 1$; *E. gallinarum*, $n = 2$; *E. faecium*, $n = 1$; and *E. faecalis*, $n = 4$).

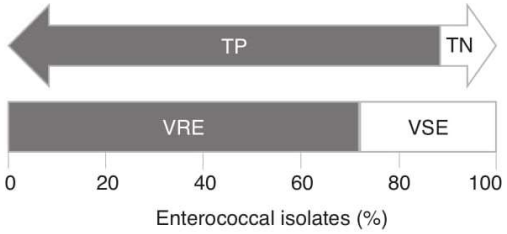
All isolates were previously identified by phenotypic methods (hydrolysis of esculin in the presence of bile, production of pyrrolidonyl arylamidase, growth in broth containing 6.5% NaCl, and negative catalase test evidentiated by the absence of effervescence).[3] Polymerase chain reaction was also used to confirm the presence of the genus *Enterococcus* and distinguish the species according to methods previously described by Ke et al.[5] and Karyama et al.[4] All isolates were obtained from the culture collection of the Gram-positive Cocci Laboratory – UFCSPA and stored in skim milk (Difco™) at −20 °C. Vancomycin minimum inhibitory concentration was determined by broth microdilution according to CLSI guidelines (2015)[6] and by Etest® according to the manufacturer's instructions. The chromID™ VRE (bioMérieux, Brazil S/A) assays were performed in two steps. First, the isolates that were previously stored in skim milk were grown in bile-esculin agar to confirm the presence of enterococci and check culture purity. Second, pure samples were grown in trypticase soy agar for 24 h, followed by single-colony growth in chromID™ VRE agar at 37 °C. After 24 h, plates with any growth were considered VRE positive. A negative result was defined as a 48-h incubation period

* *Corresponding author.*
E-mail: reosoares@gmail.com (R.O. Soares).
http://dx.doi.org/10.1016/j.bjm.2017.03.005

Table 1 – Clinical isolates used to evaluated the performance of the chromID™ VRE.

Species	Susceptibility to vancomycin[a]		Origin	
	S	R	Infection	Surveillance culture
E. faecalis	43	47	47	43
E. faecium	7	87	75	19
Total	50	134	122	62

[a] S, susceptible; R, resistant.



TP = Test positive, TN = Test negative

Fig. 1 – Schematic representation of the results obtained using chromID™ VRE.

without any bacterial growth. According to the manufacturer's instructions, chromID™ VRE agar allows the identification of species based on the detection of enzyme activity. Therefore, *E. faecium* was stained purple and *E. faecalis* was stained blue-green.

Of the *E. faecalis* VRE tested ($n=47$), 41 were stained blue-green, 5 were stained gray, and 1 isolate did not grow. All *E. faecium* VRE tested ($n=87$) were stained purple. Among VSE isolates ($n=50$), 15 did not grow (10 *E. faecalis* and 5 *E. faecium*) and 35 (33 *E. faecalis* and 2 *E. faecium*) showed some growth at the edges of the plates in the corresponding color of each species, which may suggest false-positive results. All VSE-reference strains tested ($n=8$) did not show any visible growth in the chromogenic medium. Fig. 1 shows a schematic representation of the results obtained with chromID™ VRE agar.

The chromID™ VRE agar had a sensitivity of 87.23% and 100% for detecting *E. faecalis* VRE and *E. faecium* VRE, respectively, and a combined sensitivity and specificity of 95.52% and 30.00%, respectively, for detecting VRE. No difference was observed in the specificity and sensitivity at 24 and 48 h. The positive predictive value (corresponding to the percentage of VRE that tested positive in chromID™ VRE) was 78.53% (95% confidence intervals; C.I. = 72.22–84.83%) and the negative predictive value was 71.43% (95% C.I. = 52.10–90.75%) (Table 2). Regarding sensitivity, similar results have been obtained in previous studies evaluating the performance of chromID™ VRE.[1,7–14] In relation to the specificity, previous studies have obtained values higher than 95%,[1,7,8,11] in contrast to the low specificity observed in this study.

Colonies with non-discriminatory staining (gray, dark, or colorless) have been reported in some studies.[10,15] We also observed VRE colonies with a grayish shade, which may lead to false-negative results.

Among all VRE isolates, 100% of the *E. faecium* grew within 24 h. The same was observed in previous studies.[11,14] According to Grabsch et al.,[8] 24-h identification of VRE allows earlier confirmation of colonization by these strains, facilitates infection control, and helps to avoid the spread of microorganisms. However, one *E. faecalis* VRE isolate did not grow even after 48 h, which may suggest that some strains can exhibit a different behavior and/or require more time to grow in the medium.

Delmas et al.[1] compared growth before and after an enrichment step in bile-esculin agar supplemented with vancomycin in order to select only VRE strains. The enrichment step improved the performance of chromID™ VRE at 24 h of incubation. Other studies have shown that strains incubated overnight in an enrichment broth containing vancomycin as a first step followed by the use of chromID™ VRE resulted in improved specificity or sensitivity.[1,10,11,14,16] However, this method is only useful for fecal specimens due to the large number of different microorganisms that can be present in these samples.

Most studies evaluating chromID™ VRE performance have used only fecal specimens (stool samples and rectal swabs) or only resistant strains. In our study, we evaluated well-characterized vancomycin-resistant and -susceptible isolates in order to observe the possible occurrence of false-positive results, because incorrectly prescribed antibiotics have a negative clinical impact. Despite the data presented here, a possible limitation of this study is that fecal samples were not included,

Table 2 – Evaluation of chromID™ VRE in detecting true positive vancomycin-resistant enterococci.

ChromID™ VRE agar	Gold standard (VRE)[a]	Gold standard (VSE)[a]	Total
Test positive	128	35	163
Test negative	6	15	21
Total	134	50	184
Sensitivity		95.52% (95% C.I. = 92.02–99.02%)	
Specificity		30.00% (95% C.I. = 17.29–42.70%)	
Positive predictive value		78.53% (95% C.I. = 72.22–84.83%)	
Negative predictive value		71.43% (95% C.I. = 52.10–90.75%)	
Accuracy		77.72% (95% C.I. = 71.70–83.73%)	

[a] Vancomycin resistance was determined using both Etest® and CLSI guidelines; C.I., confidence intervals.

because the density of microorganisms in a clinical specimen may affect the correct diagnosis.

In conclusion, the chromID™ VRE agar is a rapid and useful tool for the screening and identification of VRE, with a good sensitivity of about 96.00%. However, because specificity (30.00%) was limited by false positive VRE mainly, we recommend further VRE identification by conventional tests to avoid misinterpretation.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This study was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Universidade Federal de Ciências da Saúde de Porto Alegre. We also thank the bioMérieux, Brazil S/A for the supply of the chromID™ VRE agar plates.

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4.2 HIGH DIVERSITY OF VANCOMYCIN-RESISTANT *Enterococcus faecium* ISOLATED IN SOUTHERN BRAZIL

Renata Oliveira Soares[1*], Gabriela Rosa da Cunha[1], Vinicius Pieta Perez [2], Jussara Lopes Siqueira[1], Mariana Sales[1], Guy Caljon[3], Juliana Caierão [4], Paul Cos[3], Pedro Alves d'Azevedo[1]

[1]Federal University of Health Sciences of Porto Alegre, Laboratory of Gram Positive Cocci, Porto Alegre, Brazil

[2]Federal University of Paraíba, Physiology and Pathology Department, João Pessoa, Brazil

[3]University of Antwerp, Laboratory for Microbiology, Parasitology and Hygiene, Antwerp, Belgium

[4]Federal University of Rio Grande do Sul, School of Pharmacy, Department of Analysis, Porto Alegre, Brazil

*Short communication will be submitted to *Journal of Infection, Genetics and Evolution*

Impact Factor: 2016: 2.885

Abstract

Vancomycin-resistant enterococci (VRE) is common in some hospital settings and their clonal spread has been described in different regions of the world. We determined the antimicrobial susceptibility profile and the clonal relationship of VRE isolates recovered from inpatients attended at three general Hospitals of Porto Alegre, Brazil. Ninety-four VRE were characterized as *Enterococcus faecium* and exhibited resistance to teicoplanin, ampicillin, ciprofloxacin, and susceptibility to linezolid, quinupristin-dalfopristin and daptomycin. HLR-Ge was detected in 13.8% of them. All VRE_{fm} harbored *vanA* gene, while 85.1% and 94.7% harbored, respectively, *esp* and *acm* virulence genes. PFGE profile analysis revealed 23 clonal types including 79 isolates, while 15 isolates exhibited unique pattern type, showing a polyclonal distribution of VRE_{fm} in Southern Brazil. These findings contribute to the local understanding regarding the characteristics of the circulating VREs in the region.

Key-words: VRE, *acm* gene, *esp* gene, PFGE, clonal types

Introduction

It is well-stablished the remarkable ability of *Enterococcus faecium* to rapidly acquire mobile genetic elements associated to antimicrobial resistance (Gilmore et al., 2013; Cattoir and Giard, 2014; García-Solache et al., 2016). Vancomycin-resistant *Enterococcus faecium* (VRE_{fm}) has become increasingly common in some hospital settings and their clonal spread has been described worldwide (Freitas et al., 2016; Mahony et al., 2018), including Brazil (Alves et al., 2017; Resende et al., 2014; Sacramento et al., 2017). Most VRE_{fm} isolated from Brazilian Hospitals belongs to clonal complex 17 (CC-17), a well-adapted lineage to the hospital environment and responsible to the major of VRE_{fm} infections worldwide (Top et al., 2008; Palazzo et al., 2011; Alves et al., 2017; Sacramento et al., 2017). VanA-related VRE_{fm}, the most prevalent phenotype around the world, frequently presents virulence factors which facilitates the infection process, as well as multirresistance features, which considerably reduces therapeutical choices (Ahmed and Baptiste, 2017).

The aim of this work was to determine genetic relatedness of VRE_{fm}, focusing on virulence and resistance characteristics.

Materials and Methods

Bacterial Strains

Ninety-four vancomycin resistant enterococci from the Gram-Positive Laboratory Microrganism Bank (Federal University of Health Sciences of Porto Alegre, Brazil) were evaluated. The isolates were recovered from clinical samples of patients attended in hospitals of Porto Alegre, Brazil, from September/2012 to April/2017, as part of an epidemiological surveillance study. Only one isolate per patient was

considered. The project was approved by the Ethics Committee of Human Research of Federal University of Health Sciences of Porto Alegre, under the number 1.283.544.

Identification of *Enterococcus* species, vancomycin resistance and virulence genes

Primary genus identification was performed through the observation of specific phenotypic characteristics by the respective hospital's microbiology laboratory. The genus confirmation, species identification, detection of the vancomycin resistant determinants *vanA* and *vanB* genes and virulence genes *acm* (adhesin of collagen) and *esp* (enterococcal protein surface) were determined by PCR as previously described (Kariyama et al., 2000; Rathnayake et al., 2012; Kafil and Mobarez, 2015;). Primers used in this study are described in Supplementary Table 1.

Antimicrobial Susceptibility testing

The antimicrobial susceptibility profile was done using disk diffusion for ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (120 µg), linezolid (30 µg), quinupristin-dalfopristin (15 µg) and teicoplanin (30 µg) and interpreted according to CLSI 2017 guidelines (Clinical and Laboratory Standards Institute, 2017). *Minimum Inhibitory Concentrations* (MIC) was determined by Etest® strips (bioMeriéux) for daptomycin. Multidrug resistance (MDR) strains were defined as those presenting resistance to three or more different antimicrobial classes (Magiorakos et al., 2012). *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 were used as quality control.

Chromosomal Analysis of Genomic DNA by PFGE

Pulsed-field gel electrophoresis (PFGE) was performed as previously described (Saeedi et al., 2002), with the following modifications: agarose plugs were prepared and treated with 1 mg/mL of lysozyme (Sigma Co., 48000U/mg), 5U/mL of mutanolysin (Sigma Co., 3000U/mL). Digestion of chromosomal DNA was achieved with 20 U of Anza™ 22 *SmaI* (Thermo Fisher Scientific®) and restriction fragments were separated using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA).

Results were analyzed with Bionumerics software version 7.1 (Applied Maths) using the unweighted-pair group method with arithmetic mean (UPGMA). Dendrogram was constructed using dice coefficients with optimization and tolerance set to 0.5% and 1%, respectively. Clustering above 80% similarity were considered as a clone type (CT) (Alves et al., 2017).

Results

All 94 Vancomycin-Resistant Enterococci were identified as *Enterococcus faecium.* Enteroccci were recovered from urine 42.6% (n=40), blood 29.8% (n=28), rectal swab 14.9% (n=14), body fluids 11.7% (n=11) and catheter 1.1% (n=1).

All VRE_{fm} exhibited vancomycin MICs higher than 256 μg/mL and resistance to teicoplanin (all carrying *vanA* gene). They were resistant to ampicillin, ciprofloxacin, and susceptible to linezolid, daptomycin (MIC ≤ 4) and quinupristin-dalfopristin. High-Level Resistance to Gentamicin was detected in 13 (13.8%) isolates.

Considering virulence genes, 80 (85.1%) and 89 (94.7%) isolates harbored *esp* and *acm* genes, respectively. Seventy-six isolates carried both genes and one isolate did not possess any of the those.

PFGE defined 23 clone types (CTs) which included 79 of the 94 isolates, and 15 were singletons (Figure 1, Table 1). There was one dominant cluster, CT8, including 17,7% of VRE_{fm}, recovered either from infection (blood, urine) and surveillance cultures.

Discussion

VRE_{fm} has become one of the leading causes of nosocomial infections, especially among severely ill patients (Howden et al., 2013). We described the clonal relationship of 94 VRE_{fm} recovered from inpatients in Porto Alegre, Southern Brazil. Besides vancomycin, all *E. faecium* exhibited resistance to ampicillin and ciprofloxacin, and 13.8% high level resistance to gentamicin.

Around the world, studies have reported the spread of CC-17 (Alves et al., 2017; Brilliantova et al., 2010; López et al., 2012; Palazzo et al., 2011), a lineage that exhibits resistance to most antibiotics clinically used for the treatment of enterococcal infections, is well adapted to the hospital environment and has been associated with most of the reported hospital outbreaks worldwide (Panesso et al., 2010; Willems et al., 2005). Our isolates showed phenotypic characteristics similar to the CC-17 lineage, such as ampicillin and ciprofloxacin resistance and presence of *esp* gene (Gao et al., 2018). Indeed, most VRE_{fm} harboured *esp* and *acm* genes, both related with biofilm formation and adherence to extracellular matrix, giving *E. faecium* selective advantages in the hospital environment (Hendrickx et al., 2007).

Similar to our findings, Akpaka et al., (2017) performed a study between 2009 to 2014 with twelve hospitals from eight Caribbean countries and they found 31.4% of VRE strains. Among these, 70 were *E. faecium*, harboring *vanA* and *esp* genes, with 100% of resistance to ciprofloxacin, 92.8% resistance to ampicillin and 100% of susceptibility to daptomycin, linezolid and quinupristin/dalfopristin.

In a study performed in 2011 evaluating antimicrobial susceptibility patterns of isolates from 11 countries in Latin America, Brazil presented the highest rate of VRE (27%) (Jones et al., 2013). In 2016, a SENTRY study reported a rate of 71.7% of VRE_{fm} in Brazil (Sader et al., 2016).

Although *E. faecalis* is more prevalent in enterococcal infection, VRE_{fm} has been increasing in Brazilian hospitals. Conceição et al. (2011), observed an increase of 13% in VRE rate in a hospital in southeastern Brazil, between 2006-2009, being 89.5% *vanA-E. faecium*. Another study conducted with 29 isolates from a hospital in southern Brazil observed that all isolates were VRE_{fm} carrying *vanA* gene and were part of a main clone (Resende et al., 2014).

In our study, VRE_{fm} were classified into 38 types (23 clonal types and 15 singletons), demonstrating a high genetic heterogeneity. A similar polyclonal distribution of VRE_{fm} have also been observed in other studies (Landerslev et al., 2016; Pourshafie et al., 2008; Somily et al., 2016; J. Top et al., 2008).

Finally, our study contributes to the local understanding about the characteristics of the circulating VREs in the region, once there are few publications on this topic in the last 5 years in Brazil.

Acknowledgements

This study was supported in part by Research Support Foundation of the State of Rio Grande do Sul (FAPERGS), Coordination for the Improvement of Higher Education Personnel (CAPES) and Federal University of Health Sciences of Porto Alegre (UFCSPA).

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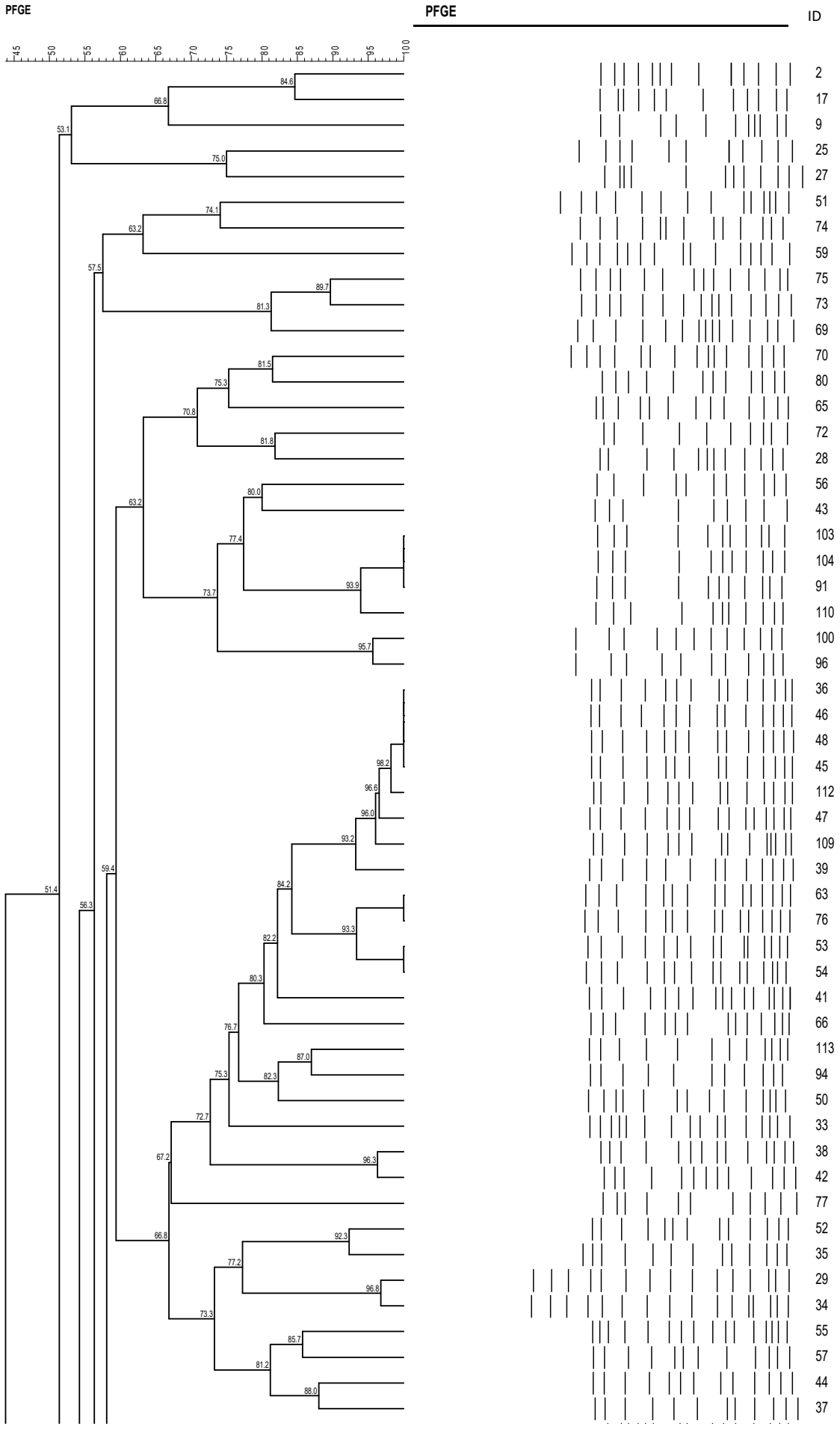
Supplementary Table 1. Description of primers used in PCR for the detection of species and vancomycin-resistance genes and virulence factors of *Enterococcus faecium*:

Target gene	Sequence of primer	Amplicon Size (pb)	Reference
Enterococcus faecium	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	658	[18]
Enterococcus faecalis	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	941	[18]
vanA	5'-CATGAATAGAATAAAAGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	1030	[18]
vanB	5'-GTGACAAACCGGAGGCGAGGA-3' 5'-CCGCCATCCTCCTGCAAAAAA-3'	433	[18]
esp	5'-GGAACGCCTTGGTATGCTAAC-3' 5'-GCCACTTTATCAGCCTGAACC -3'	95	[17]
acm	5'-GGCCAGAAACGTAACCGATA-3' 5'-AACCAGAAGCTGGCTTTGTC-3'	135	[26]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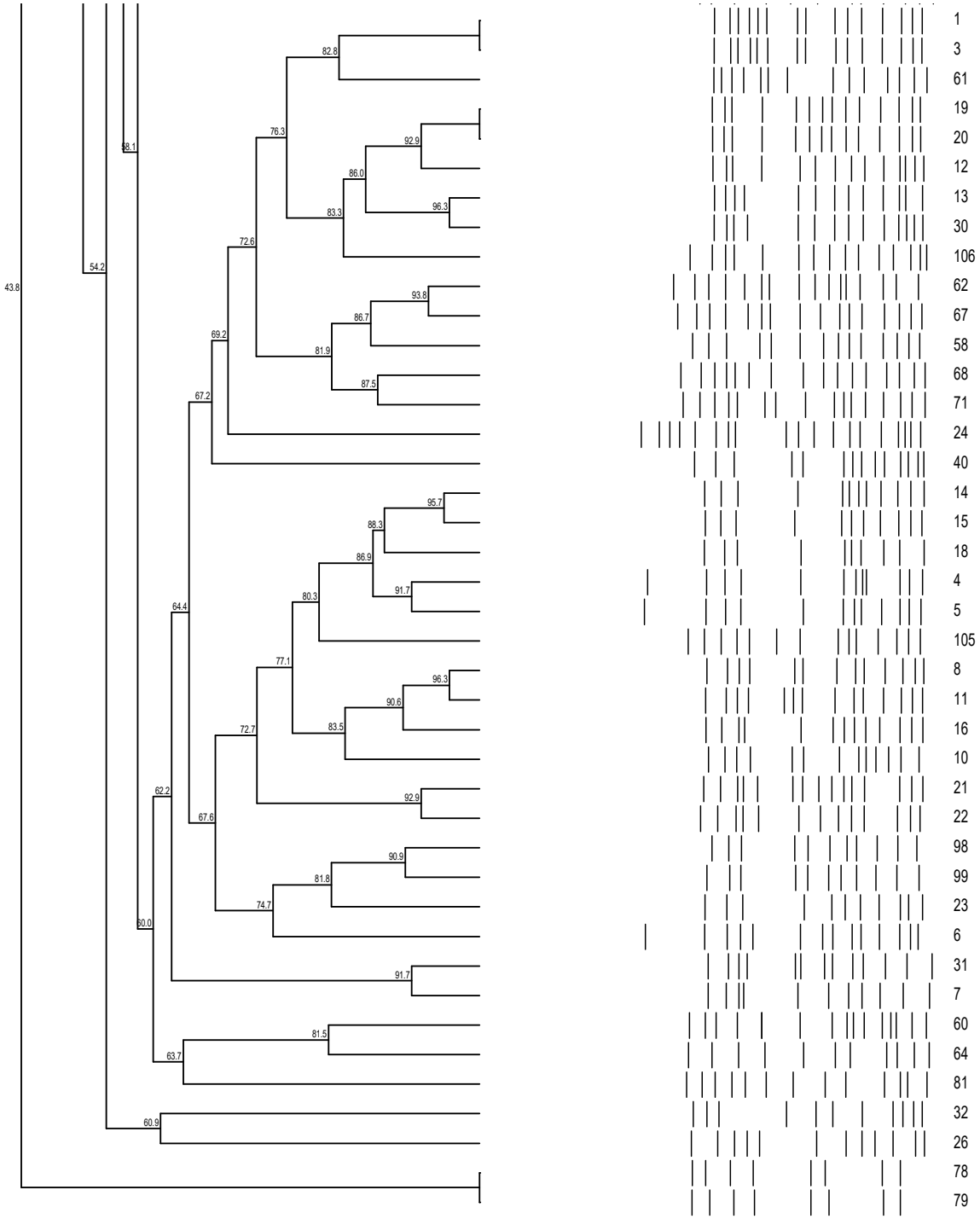

Fig.1 PFGE dendrogram and PFGE profile images of 94 *vanA E. faecium* from Porto Alegre, Brazil.

Table 1. Description of 94 VRE_{fm} clinical isolates from Porto Alegre, Brazil, recovered from Sept-2012 to Apr-2017.

Strain ID	Source	Date	PFGE	Resistance Profile	Virulence Profile
2	Urine	Oct-12	CT1	AMP, CIP, TEI, VAN	*esp+, acm+*
17	Urine	May-13	CT1	AMP, CIP, TEI, VAN	*esp+, acm+*
69	Blood	Dec-16	CT2	AMP, CIP, TEI, VAN	*esp+, acm+*
73	Urine	Jan-17	CT2	AMP, CIP, TEI, VAN	*esp+, acm+*
75	Urine	Jan-17	CT2	AMP, CIP, TEI, VAN	*esp+, acm+*
70	Body Fluids	Dec-16	CT3	AMP, CIP, TEI, VAN	*esp+*
80	Urine	Apr-17	CT3	AMP, CIP, TEI, VAN	*acm+*
28	Urine	May-15	CT4	AMP, CIP, TEI, VAN	*esp+, acm+*
72	Urine	Jan-17	CT4	AMP, CIP, TEI, VAN	*esp+, acm+*
43	Body Fluids	Sep-15	CT5	AMP, CIP, TEI, VAN	*esp+, acm+*
56	Body Fluids	Mar-16	CT5	AMP, CIP, TEI, VAN	*esp+, acm+*
91	Rectal Swab	Sep-14	CT6	AMP, CIP, TEI, VAN	*esp+, acm+*
103	Rectal Swab	Dec-14	CT6	AMP, CIP, TEI, VAN	*esp+, acm+*
104	Rectal Swab	Jan-15	CT6	AMP, CIP, TEI, VAN	*esp+, acm+*
110	Rectal Swab	Jun-15	CT6	AMP, CIP, TEI, VAN	*esp+, acm+*
96	Urine	Nov-14	CT7	AMP, CIP, TEI, VAN	*esp+, acm+*
100	Rectal Swab	Dec-14	CT7	AMP, CIP, TEI, VAN	*esp+, acm+*
36	Blood	Aug-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
39	Blood	Aug-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
41	Blood	Sep-15	CT8	AMP, CIP, TEI, VAN	*esp+*
45	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
46	Urine	Nov-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
47	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
48	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
53	Urine	Jan-16	CT8	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
54	Blood	Jan-16	CT8	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
63	Blood	Oct-16	CT8	AMP, CIP, TEI, VAN	-
66	Blood	Nov-16	CT8	AMP, CIP, TEI, VAN	*esp+*
76	Urine	Jan-17	CT8	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
109	Rectal Swab	May-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
112	Rectal Swab	Jul-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
50	Body Fluids	Dec-15	CT9	AMP, CIP, TEI, VAN	*acm+*
94	Urine	Nov-14	CT9	AMP, CIP, TEI, VAN	*esp+, acm+*
113	Rectal Swab	Aug-15	CT9	AMP, CIP, TEI, VAN	*esp+, acm+*
38	Urine	Aug-15	CT10	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
42	Urine	Sep-15	CT10	AMP, CIP, TEI, VAN	*esp+, acm+*
35	Urine	Jul-15	CT11	AMP, CIP, TEI, VAN	*esp+, acm+*
52	Urine	Dec-15	CT11	AMP, CIP, TEI, VAN	*esp+, acm+*
29	Urine	May-15	CT12	AMP, CIP, TEI, VAN	*esp+, acm+*
34	Urine	Jul-15	CT12	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*

37	Urine	Aug-15	CT13	AMP, CIP, TEI, VAN	*esp+, acm+*
44	Urine	Sep-15	CT13	AMP, CIP, TEI, VAN	*esp+, acm+*
55	Urine	Jan-16	CT13	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
57	Urine	Apr-16	CT13	AMP, CIP, TEI, VAN	*esp+, acm+*
1	Blood	Sep-12	CT14	AMP, CIP, TEI, VAN	*acm+*
3	Blood	Oct-12	CT14	AMP, CIP, TEI, VAN	*esp+, acm+*
61	Body Fluids	Nov-16	CT14	AMP, CIP, TEI, VAN	*esp+, acm+*
12	Catheter	Apr-13	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
13	Blood	May-13	CT15	AMP, CIP, TEI, VAN	*acm+*
19	Urine	Jun-13	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
20	Blood	Jul-13	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
30	Blood	May-15	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
106	Rectal Swab	Mar-15	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
58	Urine	Mar-16	CT16	AMP, CIP, TEI, VAN	*esp+, acm+*
62	Urine	Oct-16	CT16	AMP, CIP, TEI, VAN	*acm+*
67	Urine	Nov-16	CT16	AMP, CIP, TEI, VAN	*esp+, acm+*
68	Urine	Dec-16	CT16	AMP, CIP, TEI, VAN	*acm+*
71	Urine	Dec-16	CT16	AMP, CIP, TEI, VAN	*acm+*
4	Urine	Oct-12	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
5	Blood	Oct-12	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
14	Blood	May-13	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
15	Body Fluids	May-13	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
18	Blood	May-13	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
105	Rectal Swab	Jan-15	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
8	Urine	Feb-13	CT18	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
10	Urine	Mar-13	CT18	AMP, CIP, TEI, VAN	*esp+, acm+*
11	Urine	Apr-13	CT18	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
16	Blood	May-13	CT18	AMP, CIP, TEI, VAN	*acm+*
21	Blood	Jul-13	CT19	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
22	Urine	Jul-13	CT19	AMP, CIP, TEI, VAN	*esp+, acm+*
23	Rectal Swab	Jul-13	CT20	AMP, CIP, TEI, VAN	*esp+, acm+*
98	Rectal Swab	Nov-14	CT20	AMP, CIP, TEI, VAN	*esp+, acm+*
99	Rectal Swab	Dec-14	CT20	AMP, CIP, TEI, VAN	*esp+, acm+*
7	Blood	Dec-12	CT21	AMP, CIP, TEI, VAN	*esp+, acm+*
31	Blood	May-15	CT21	AMP, CIP, TEI, VAN	*esp+, acm+*
60	Body Fluids	May-16	CT22	AMP, CIP, TEI, VAN	*acm+*
64	Urine	Oct-16	CT22	AMP, CIP, TEI, VAN	*esp+, acm+*
78	Blood	Feb-17	CT23	AMP, CIP, TEI, VAN	*esp+, acm+*
79	Blood	Apr-17	CT23	AMP, CIP, TEI, VAN	*esp+, acm+*
6	Blood	Dec-12	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
9	Body Fluids	Feb-13	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
24	Body Fluids	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
25	Blood	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
26	Urine	Aug-13	*Singleton*	HLG, AMP, CIP, TEI, VAN	*acm+*
27	Rectal Swab	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*acm+*

32	Urine	May-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
33	Urine	May-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
40	Blood	Sep-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
51	Blood	Dec-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
59	Urine	Apr-16	*Singleton*	AMP, CIP, TEI, VAN	*acm+*
65	Urine	Oct-16	*Singleton*	AMP, CIP, TEI, VAN	*acm+*
74	Body Fluids	Jan-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+*
77	Urine	Feb-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
81	Body Fluids	Apr-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*

PFGE, pulsed-field gel electrophoresis; CT, clonal type; HLG, high level of gentamicin; AMP, ampicillin; CIP, ciprofloxacin; TEI, teicoplanin; VAN, vancomycin; ***esp***, enterococcal protein surface gene; ***acm***, collagen adhesin gene.

4.3 INFLUENCE OF GLUCOSE SUPPLEMENTATION ON BIOFILM FORMATION AND EXPRESSION OF ASSOCIATED-BIOFILM GENES IN *Enterococcus faecalis* CLINICAL ISOLATES*

Renata Oliveira Soares[1], Adriana Medianeira Rossato[1], Dérik Midon Martins[1], Guy Caljon[2], Juliana Caierão[3], Pedro Alves d'Azevedo[1], Paul Cos[2]

[1]Federal University of Health Sciences of Porto Alegre, Laboratory of Gram Positive Cocci, Porto Alegre, Brazil

[2]University of Antwerp, Laboratory for Microbiology, Parasitology and Hygiene, Antwerp, Belgium

[3]Federal University of Rio Grande do Sul, Faculty of Pharmacy, Analysis Department, Porto Alegre, Brazil

*Short communication will be submitted to *Microbial Pathogenesis journal*

Impact factor: 2016: 2.009

Abstract

Enterococcus faecalis is one important cause of medical device-associated infections. Hence, it is crucial to understand the mechanisms of enterococcal biofilm formation. It is known that glucose and expression of different virulence genes could affect the enterococci biofilm formation. We evaluated the ability to form biofilm of 123 *E. faecalis* clinical strains in the presence and absence of 1% D-(+)-glucose. Real-time quantitative PCR was performed to evaluate the relative expression of biofilm related genes (*ebpA, efaA, ace* and *gelE*) in eleven clinical isolates and *E. faecalis* 29212 at the same conditions. It was observed that 1% glucose supplementation increased significantly the biofilm capability, however the gene expression varied among clinical isolates, showing that patterns of virulence gene expression are dependent on the bacterial isolate.

Keywords: *Enterococcus faecalis,* biofilm, virulence genes, glucose

Abbreviations: TSB, Tryptic Soy Broth; DPBS, Dulbecco's Phosphate-Buffered Saline; PCR, Polymerase Chain Reaction; CV, Crystal Violet; NC, Negative Control; SD, Standard Deviation; ODc, Optical density cut-off

1. Introduction

Enterococci are natural inhabitants of the human gastrointestinal tract; however, they are also able to cause a variety of infections, like urinary tract infections, bacteraemia, endocarditis and medical device-associated infections. Enterococci can form biofilms allowing them to survive under difficult conditions

and to tolerate more easily antibacterial treatments [1,2]. Furthermore, *Enterococcus faecalis* can harbour different virulence genes that may play a crucial role in the pathogenesis and biofilm formation. The regulation of these genes during biofilm formation is not totally understood [3]. Endocarditis and biofilm-associated pilus (*ebpA*); *E. faecalis* endocarditis antigen (*efaA*); Collagen-binding adhesin (*ace*) and Gelatinase (*gelE*) are some of the enterococcal virulence genes that have an important role in the biofilm development [4–7].

Nutritional conditions can also influence the *in vitro* biofilm formation. Glucose is a supplement that have been evaluated for biofilm formation by different microorganisms once it could increase the pathogenicity of different microbes [8–11]. Besides, there are several studies associating the presence of virulence genes with the ability to form biofilms in *E. faecalis,* as well as different essential conditions for biofilm formation [12–14]. However, to our knowledge, this is the first study to report the influence of 1% glucose on the expression of *ebpA, efaA, gelE* and *ace* genes in *E. faecalis* biofilm.

Thus, the aim of this study was to quantify *E. faecalis* biofilm formation in the presence and absence of 1% D-(+)-glucose and to evaluate the influence of 1% glucose in biofilm-related genes expression.

2. Materials and methods

2.1 Bacterial Strains

One hundred and twenty-three clinical isolates of *Enterococus faecalis* obtained from the collection of the Laboratory of gram positive cocci were selected for this study. The origins of the isolates were as follows: 95 (77.24%) from urine, 19 (15.45%) from blood and 9 (7.32%) from body fluids. All isolates were previously

51 classified as biofilm formers by Soares et al. [15]. *E. faecalis* ATCC 29212,
52 purchased from the American Type Culture Collection (ATCC®, USA), was used in
53 all assays as reference strain.
54
55 2.2 Virulence genes
56 The presence of four virulence genes, *ebpA* (5′-
57 AAAAATGATTCGGCTCCAGAA-3′ and 5′- TGCCAGATTCGCTCTCAAAG -3′),
58 *efaA* (5′- TGGGACAGACCCTCACGAATA -3′ and 5′-
59 CGCCTGTTTCTAAGTTCAAGCC -3′), *ace* (5′-
60 GGAGAGTCAAATCAAGTACGTTGGTT -3′ and 5′-
61 TGTTGACCACTTCCTTGTCGAT -3′) and *gelE* (5′-
62 TATGACAATGCTTTTTGGGAT -3′ and 5′- AGATGCACCCGAAATAATATA -3′)
63 were investigated by PCR as previously described [4–6,16].
64
65 2.3 Biofilm formation assays
66 The biofilm assays were performed in 96-well cell culture microtiter plates
67 (KASVI®). Briefly, bacterial suspensions in tryptic soy broth (TSB, KASVI®)
68 supplemented with and without 1% D-(+)-glucose (MERCK®) were added to each
69 well (8 wells per isolate). Plates were covered and incubated for 24h at 35°C under
70 static conditions. Biofilm quantification was performed according to adapted
71 protocol from O'Toole [17] and Stepanovic et al. [18] through the quantification of
72 total biomass with 0.01% crystal violet (CV) staining (Merck®). The absorbance
73 was measured in octuplicate, in a microtiter plate reader (KASUAKI) at 492 nm. *E.*
74 *faecalis* ATCC 29212 was used as positive control and non-bacterial-inoculated
75 wells were used as negative control. For each strain and negative control (NC),

average ($\overline{X}$) and standard deviation (SD) values were calculated from the OD492 values obtained in the absence and presence of glucose 1%. Optical density cut-off (ODc) was calculated by the formula:

$$\overline{X}_{NC} + 3\,(SD_{NC})$$

2.4 Biofilm induction for gene expression assays

Eleven isolates of *E. faecalis* and one control (*E. faecalis* ATCC 29212), harbouring all the four virulence genes, were randomly selected for gene expression assays. Biofilm induction was performed in 6-well microplates (Greiner CELLSTAR®, flat-bottomed sterile cell culture), in a final volume of 3 mL per well, by adding 300 µl of inoculum (1.5 x 10^8 CFU/mL). One treatment was tested per isolate: 1) Control: only TSB medium; 2) Treatment, TSB medium plus 1% D-(+)-glucose. All biofilm assays were performed in triplicate. The microplates were incubated at 35ºC for 8 hours, achieving the mid-log phase of growth. After that, the TSB medium was removed and the wells were washed twice with Dulbecco's phosphate-buffered saline (DPBS 1X, Gibco®) for removal of the non-attached cells. One mililiter of deionized water (Sigma-Aldrich®) was added to the well, and the attached cells were harvested with a cell scraper (240 mm, TPP®). Then, 500 µl of the suspension was transferred into a sterile microtube of 2mL, followed by vigorous vortexing and sonication to break up cells aggregation. One mililiter of RNAprotect Bacteria Reagent (Qiagen®) was added into a microtube for stabilization of RNA prior to RNA isolation procedures. After the RNA stabilization, bacterial cells were pelleted by centrifugation (10 min at 5000g). The supernatant was decanted and the pellet was frozen at -20ºC until the RNA extraction.

101 2.5 RNA extraction and cDNA synthesis

102 RNA was extracted by enzymatic lysis using lysozyme (Sigma-Aldrich®), and
103 proteinase K (Sigma-Aldrich®) digestion followed by purification using the RNeasy
104 Mini Kit (Qiagen®), according to manufacturer procedures. After the extraction, the
105 amount of the total RNA extracted was determined using the Thermo Scientific
106 NanoDrop™ 2000 Spectrophotometer, as the 260nm/280nm ratio with expected
107 values between 1.8 and 2.

108 The amount of 40 ng of high-quality total RNA was reverse-transcribed to
109 complementary DNA (cDNA) in 10 µl volume using the AccuScript High Fidelity
110 RT-PCR System (Agilent Technologies) according the manufacturer's instructions.
111 After that, cDNAs were diluted (1:20) and frozen at -20ºC until qPCR reaction.

112

113 2.6 Reference genes and target genes

114 Two reference genes and four virulence genes were chosen for the
115 expression assays. The elongation factor Tu, a GTP binding protein involved in
116 peptide chain formation, encoded by *tuf* gene [19] and the 23S Ribossomal RNA,
117 part of the large 50S subunit of the ribosome in prokaryotes, encoded by *23SrRNA*
118 gene [20] were selected as reference genes. Primers for reference genes *tuf* (5′-
119 ATTAATGGCTGCAGTTGACG -3′ and 5′- AGCAACAGTACCACGTCCAG -3′) and
120 *23SrRNA* (5′- CAGTGTCAGATGGGCAGTTT -3′ and 5′-
121 GCTCCCTTCTGCCTTTACAC -3′) were obtained using the Primer3Plus software
122 version: 2.4.0 [21]. Primers specificity was evaluated *in silico* with genome of
123 *Enterococcus faecalis* V583 (NC_004668.1) using the BLAST® software (National
124 Centre for Biotechnology Information).

125 The target genes used in this assay were the same evaluated in the
126 conventional PCR: *ebpA, efaA, ace* and *gelE.* PCR efficiency was evaluated for
127 each primer and the amplification efficiency (E) was calculated from the slope of
128 the standard curve using the formula $E = 10^{-1/slope}$ (ideal value between 1.9 and
129 2.1).
130
131 2.7 Quantitative real-time PCR
132 Quantitative real-time PCR was performed in the StepOnePlus™ Real-Time
133 PCR System (Applied Biosystems™). Master-mix for each PCR run was prepared
134 in 20 µl volume as follows: 10 µl SYBR® Green PCR Master Mix (Applied
135 Biosystems™), 5.6 µl water (Sigma-Aldrich®), 0.2 µl of each primer (0,1 µM) and 4
136 µl of cDNA. The following amplification program was used: 10 min of denaturation
137 at 95ºC, 40 cycles of real-time PCR consisting of 15 s at 95ºC for denaturation, 15
138 s at (56ºC to *tuf, efaA, ebp, gelE* and *ace* genes and 60ºC for *23SrRNA* gene), 45
139 s at 60ºC for annealing, and a final melting curve. All reactions were performed in
140 triplicate with two biological replicates.
141
142 2.8 Data analysis
143 Data were described as mean ± SD. Differences in the biofilm formation
144 comparing medium with and without glucose was calculated by percentage of
145 increase and decrease of the optical density. Statistical analyses were performed
146 by GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla
147 California USA, www.graphpad.com). All tests were performed with a confidence
148 level of 95%. Relative expression analysis of target and reference genes in the
149 treated-well was performed comparing to the non-treated sample, by the REST

2009 software (PFAFFL, 2001 and Qiagen) using the crossing points (CP) of each gene studied. Results of expression greater than 2-fold were considered significant.

3. Results

3.1 Effects of glucose supplementation on biofilm formation

The biofilm-forming ability of *E. faecalis* in the presence and absence of 1% glucose after 24 hours of incubation was evaluated by measuring the total biomass using CV staining. In the absence of glucose, 10 (8.1%) of the isolates were classified as non-producers, 60 (48.8%) were weak producers, 45 (36.6%) were moderate producers and 8 (6.5%) were strong producers. On the other hand, in the presence of 1% glucose, all *E. faecalis* tested were biofilm producers, being 18 (14.6%) classified as weak producers, 44 (35.8%) were moderate producers and 61 (49.6%) were strong producers (Figure 1A).

Among the 10 isolates non-biofilm formers in the absence of glucose, it was observed induction of the biofilm expression by the presence of 1% glucose, once all isolates were able to form biofilm with glucose. Considering the 18 isolates previously classified as weak biofilm formers in the presence of glucose, 5 isolates were negative and 13 were weak in the absence of this supplement. On the other hand, among the 44 isolates classified as moderate biofilm formers in the presence of glucose, 4 were negative and 30 were weak in the absence of the supplement, showing an increase in the capability to form biofilm with glucose supplementation. Finally, among the 61 isolates classified as strong biofilm formers in the presence of glucose, 6 also exhibited strong capability to form biofilm in the absence of the supplement, while the other 55 isolates were negative, weak or moderate biofilm

175 formers in the absence of glucose. It confirms the premise that this nutrient is
176 important for the expression or increase of the biofilm formation ability.
177 Figure 1B shows the OD 492 obtained in the absence and presence of 1%
178 glucose. In the presence of 1% glucose, the basal OD 492 were significantly higher
179 than those without glucose ($p<0.02$) for strong producers, representing an increase
180 of 41%. However, no significant differences were observed in the basal OD 492 in
181 the weak and moderate producers in both conditions.
182
183 3.2 Presence of the virulence genes
184 Overall, 118 (95.9%) isolates harboured at least two of the four virulence
185 genes evaluated; 42 (34.1%) isolates harboured all the four genes. Eighty-five
186 (69.1%) *E. faecalis* harboured *ebpA*, 118 (95.9%) *efaA,* 79 (64.2%) *ace* and 87
187 (70.7%) *gelE*. Among isolates from urine all genes had the highest frequency of
188 occurrence (73.7% *ebpA;* 96.8% *efaA;* 72.6% *ace* and 70.5% *gelE*), followed by
189 blood (52.6% *ebpA;* 100% *efaA;* 42.1% *ace* and 73.7% *gelE*) and body fluids
190 (55.6% *ebpA;* 77.8% *efaA;* 33.3% *ace* and 66.7% *gelE*).
191 Among the 42 isolates harbouring all the four genes evaluated in this study
192 (*ebpA, efaA, gelE and ace*) and the 46 isolates that harboured at least 3 of the
193 genes, 95.2% and 87% of the isolates, respectively, exhibited moderate or strong
194 ability to form biofilm in the presence of glucose.
195
196 3.3 Effects of glucose in *ebpA, efaA, ace* and *gelE* genes expression
197 The relative expression of virulence genes was obtained using qPCR.
198 Expression of each gene was evaluated for eleven *E. faecalis* clinical isolates and
199 one reference strain (*E. faecalis* ATCC 29212) after biofilm induction with 1%

glucose. Overall, the levels of transcription of all evaluated genes increased when 1% glucose was added to the medium. However, considering the average of gene expression of all twelve isolates (clinical strains and control), those increases were not statistically significant (Figure 2). Moreover, the source of recovery (urine, blood or other body fluid) did not influence expression of virulence genes we evaluated.

In the context of each clinical isolate, gene expression was affected by the presence of D-(+)-glucose. Only three clinical strains exhibited high levels of *epbA* transcription (> 2.6-fold up-regulation EFL5, EFL8 and EFL17) and *gelE* transcription (> 2.9-fold up-regulation – EFL8, EFL15 and EFL17) in 1% glucose. Transcription of *efaA* and *ace* were up-regulated to five (> 2.2-fold up-regulation – EFL5, EFL8, EFL12, EFL15 and EFL17) and six (> 2-fold up-regulation – EFL3, EFL5, EFL8, EFL12, EFL15 and EFL17) clinical strains, respectively (Table 1). For all other strains, no significant differences were observed in the transcription levels between the treatment and the control condition.

Comparing the biofilm formation capability in the absence or presence of 1% glucose with the biofilm-related gene expression, it was observed that 6 isolates exhibited at least one gene positively regulated by the presence of glucose and they also increased the biofilm expression in the presence of the supplement. In contrast, 4 isolates (EFL 6, EFL 7, EFL 13 and EFL 14) increased their capability to form biofilm but their biofilm-related genes expression unchanged in the presence of glucose. Finally, one isolate (EFL 4) had not alterations in the gene expression and it remained as moderate biofilm former in the presence and absence of glucose.

4. Discussion

This study evaluated the *in vitro* effects of glucose on biofilm formation. The process of biofilm formation involves different steps: attachment, accumulation, maturation and dispersal. Initially, the attachment step is reversible and starts with the adhesion of planktonic cells on a surface. After that, this adhesion becomes irreversible, the microbial cells start to growth, and form aggregates. The maturation step involves the fully biofilm formed wrapped by extracellular polymeric substance [23,24].

The presence of glucose seems to be an essential factor for biofilm formation by different bacteria, as well, contributes to bacterial growth, reproduction and metabolic activities. However, the optimal concentration varies among different publications [10,25,26]. In *E. faecalis*, biofilm formation decreases when exposed to low glucose concentrations (0.05 and 0.15%) [10], therefore, high or low glucose concentrations can influence positively or negatively the biofilm formation and thus we tested the optimal concentration. In previous experiments, we observed that 1% glucose gave the highest biofilm formation in *E. faecalis* (data not published).

Pillai et al. [27] also showed that 1% glucose supplementation increased the optical density and can regulate, directly or indirectly, the transcription of some glucose-dependent genes associated with biofilm formation in *E. faecalis*. All isolates evaluated in this study were classified before as biofilm formers in the presence of glucose. Glucose supplementation significantly increased biofilm formation already at 8 hours. Similar result was observed by Seneviratne et al. [25] that evaluated the biofilm formation of *Enterococcus faecalis* in 2% glucose as well as different culture media compositions.

Commonly, studies have shown a correlation between the presence of some virulence genes with the ability of *Enterococcus* to form biofilms [12,13,15,28]. The *efaA* gene was the most prevalent, present in 95.9% of the *E. faecalis* isolates, followed by *gelE* (70.7%), *ebpA* (69.1%) and *ace* (64.2%). Assuming that, and knowing that all isolates evaluated were biofilm formers, our results reinforces the idea that some genes may not be mandatory for the biofilm formation in enterococci, once they are not present in all strains. In a study performed with 196 isolates of enterococci from urine, it was demonstrated that the presence of *efaA* increases the biofilm formation while *ace* and *gelE* genes had not effect on biofilm production in this genre [29].

Our study also investigated the direct influence of glucose in the biofilm-related genes expression. Isolates evaluated in the expression assays possessed all the four genes (*ebpA, efaA, ace* and *gelE*). Interestingly, our findings demonstrated that some genes had not its expression altered in the presence of glucose, showing that patterns of virulence gene expression are dependent on the bacterial isolate.

The biofilm formation in *E. faecalis* involves transcriptional regulatory systems or cell-surface components [30]. Many studies prove that *epbA, efaA, ace,* and *gelE* genes are strongly associated with pathogenesis of *Enterococcus* spp*.,* even as they play an individual role in the biofilm biogenesis [31]. Meantime, it was not possible to perform a correlation between the effect of glucose supplementation on biofilm formation and on expression of the genes evaluated.

In this study, all four genes were up-regulated in 1% glucose in a subgroup of isolates and no significant differences were observed for the others. Up-regulation of *ace* gene transcription in the presence of 1% glucose was observed

in 6 (50%) of the 12 isolates of *E. faecalis* evaluated, followed by the up-regulation of *efaA* gene 5 (41.7%), *ebpA* gene 3 (25%) and *gelE* gene 3 (25%). Ran et al. [10] also evaluated the transcription levels of *ace* and *gelE* genes of *E. faecalis* ATCC 33186, in the presence and absence of glucose, and they observed up-regulation of the *ace* gene in no glucose medium and up-regulation of the *gelE* gene in 0.15% glucose. As we observe, the levels of gene expression are particular of each clinical strain. According to Nallaparedy and Murray [6], expression of *ace* may be regulated in a variety of environmental conditions by different mechanisms.

In conclusion, this study demonstrated a positive effect of 1% glucose on biofilm formation of *E. faecalis,* however significant differences in biofilm-related gene expression were observed only for some strains. Biofilm formation is a multifactorial process influenced by different factors such as gene expression and conditions of induction. A better understanding of this process may help in the search for possible targets to prevent biofilm formation by this microorganism.

Acknowledgements

This study was supported by the Brazilian National Council for Scientific and Technological Development (CNPq) and the Coordination for the Improvement of Higher Education Personnel (CAPES).

Disclosure

There are no conflicts of interest to declare.

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410

Figure and Table Legends

Fig. 1. (A) and (B) Absorbance values of CV solutions (OD492), obtained from 24 h biofilm of *E. faecalis* strains formed in the presence and absence of 1% glucose. Error bars represent the standard error. Statically differences obtained when compared with strong biofilm formed without glucose ($^{*}p < 0.05$).

Fig. 2. Level of gene expression of *ebpA*, *efaA*, *ace* and *gelE* in 8 h biofilm of *E. faecalis* with 1% glucose. The values were calculated using Pfaffl method, normalized by the expression of the housekeeping gene and compared with the genes expression without glucose. Error bars represent standard deviation.

Table 1. Gene expression rate and percentage of biofilm expression change rate after glucose supplementation (Gene expression assay and biofilm induction performed at 8 h with 1% glucose)

Fig. 1.

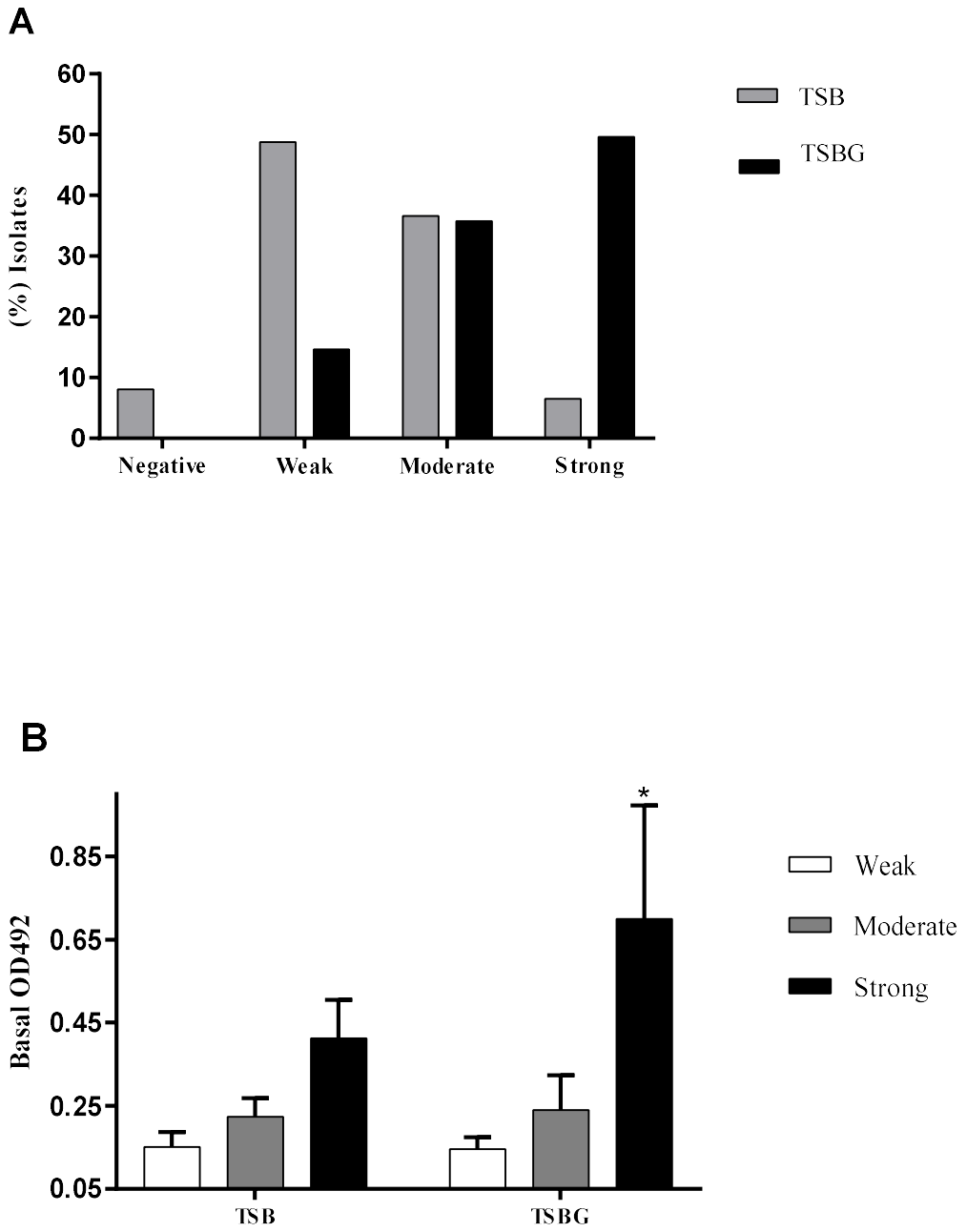
A
60
50
40
30
20
10
0
(%) Isolates
Negative
Weak
Moderate
Strong
TSB
TSBG
B
0.85
0.65
0.45
0.25
0.05
Basal OD492
*
TSB
TSBG
Weak
Moderate
Strong

Fig. 2.

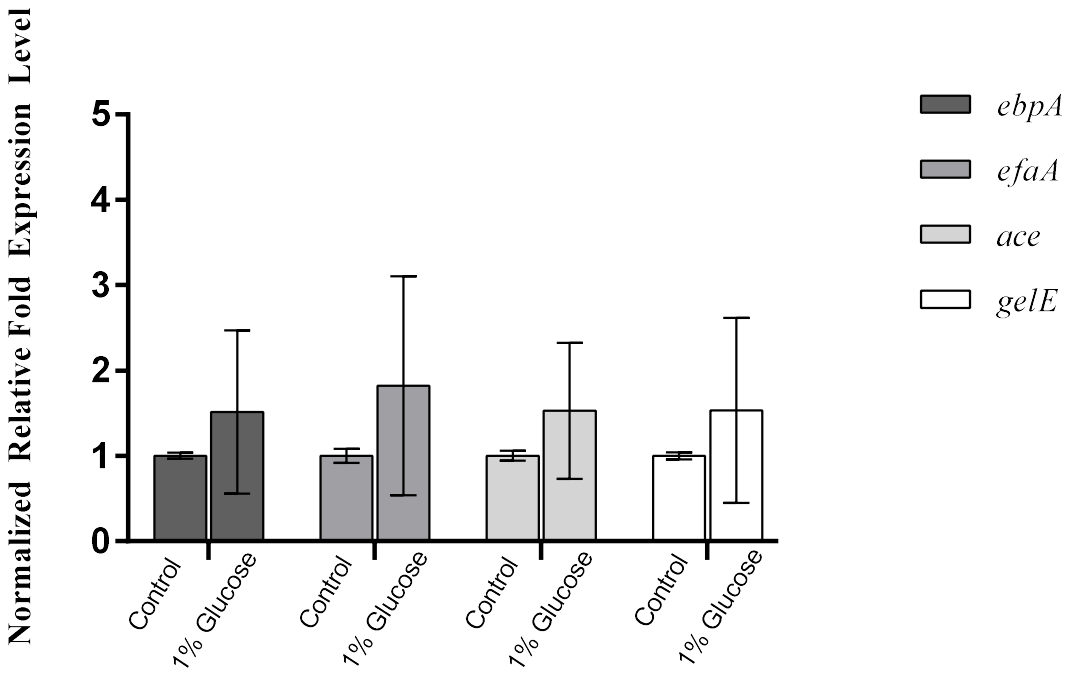
Normalized Relative Fold Expression Level
5
4
3
2
1
0
Control
1% Glucose
Control
1% Glucose
Control
1% Glucose
Control
1% Glucose
ebpA
efaA
ace
gelE

Table 1.

Isolate	Source	*ebpA*	*efaA*	*ace*	*gelE*	Biofilm**
EFL3	Body fluids	1.02 ± 0.26	1.39 ± 0.24	2.42 ± 0.57 *	1.52 ± 0.35	462%
EFL4	Body fluids	0.4 ± 0.39	0.48 ± 0.01	0.39 ± 0.02	0.42 ± 0.06	-8%
EFL5	Urine	2.96 ± 0.08 *	4.07 ± 0.18 *	2.22 ± 0.12 *	1.1 ± 0.09	413%
EFL6	Blood	1.16 ± 0.05	1.37 ±0.12	1.26 ± 0.05	1.18 ± 0.12	167%
EFL7	Blood	0.33 ± 0.02	0.51 ± 0.02	0.45 ± 0.05	1.09 ± 0.15	503%
EFL8	Urine	2.69 ± 0.09 *	2.82 ± 0.35 *	2.33 ± 0.19 *	2.93 ± 0.22 *	197%
EFL12	Blood	1.79 ± 0.24	2.27 ± 0.12 *	2.02 ± 0.12 *	1.06 ± 0.13	158%
EFL13	Urine	0.65 ± 0.03	0.44 ± 0.09	0.69 ± 0.03	0.82 ± 0.08	372%
EFL14	Blood	1.69 ± 0.08	1.67 ± 0.06	1.84 ± 0.06	1.77 ± 0.21	351%
EFL15	Body fluids	0.9 ± 0.08	2.82 ± 0.3 *	2.4 ± 0.25 *	3.07 ± 0.19 *	379%
EFL17	Blood	3.08 ± 0.06 *	3.59 ± 0.10 *	3.21 ± 0.30 *	3.52 ± 0.06 *	291%
ATCC29212	Urine***	1.39 ± 0.2	0.42 ± 0.01	1.19 ± 0.27	0.14 ± 0.11	238%

* Significant; **Pattern of biofilm formation comparing isolates without and with glucose; ***Information obtained from ATCC®, USA

4.4 HUMAN SERUM ENHANCES VIRULENCE FACTORS EXPRESSION AND INHIBITS ADHESION OF *Enterococcus faecalis* V583

Renata Oliveira Soares[1], Francine dos Santos Ramos[1], Maíra Bidart de Macedo[2], Guy Caljon[2], Juliana Caierão[3], Pedro Alves d'Azevedo[1], Paul Cos[2]

[1]Federal University of Health Sciences of Porto Alegre, Laboratory of Gram Positive Cocci, Porto Alegre, Brazil

[2]University of Antwerp, Laboratory for Microbiology, Parasitology and Hygiene, Antwerp, Belgium

[3]Federal University of Rio Grande do Sul, Faculty of Pharmacy, Analysis Department, Porto Alegre, Brazil

*Original article will be submitted as a brief note to *Journal Research in Microbiology*

Impact Factor: 2016: 2.549

Abstract

The *in vitro* effects of different concentration (1%, 5%, 25% and 50%) of human serum (HS) on biofilm formation and virulence gene expression (*ebpA, efaA, ace, gelE* and *asa*) of *E. faecalis* V583 were evaluated. No differences were observed in the planktonic growth; however, it was observed an inhibition of the adhesion step of biofilm formation in 5% HS. Planktonic cells of *E. faecalis* V583 exhibited upregulation of asa gene in 5%, 25% and 50% HS, while in the sessile cells, *efaA, gelE, ace* and *asa* genes were significantly upregulated in high concentrations of HS (25% and 50%).

Keywords: *Enterococcus faecalis,* biofilm, virulence genes, human serum

1. Introduction

Enterococci are part of the normal microbiota of humans and animals; besides, they are also recognized as an important opportunistic agent of urinary tract infections, endocarditis, bacteraemia and medical-device related infections [1,2]. Currently, 57 species of enterococci are described, being *Enterococcus faecalis* and *Enterococcus faecium* the most common species in health-care associated infections, both capable of forming biofilm and harbouring different virulence factors [3]. Endocarditis and biofilm-associated pilus (EbpA); *E. faecalis* endocarditis antigen (EfaA); Collagen-binding adhesion (Ace); Gelatinase (GelE) and Aggregation substance (Asa) are some of the enterococci virulence genes that have an important role in the complex process of biofilm development [4–7].

Serum from humans and animals is a supplement that has been evaluated for biofilm formation by different microorganisms [8–11]. Therefore, the aim of this

study was to evaluate the *in vitro* effects of human serum on *E. faecalis* V583 biofilm formation. The expression of five virulence genes was analysed for both planktonic and sessile cells of *E. faecalis* V583.

2. Materials and Methods

2.1 Bacterial strain and growth conditions

Enterococcus faecalis V583 (ATCC 700802), from human blood, was purchased from the American Type Culture Collection (ATCC®, USA) and stored at -20ºC in Mueller-Hinton broth (MHB, KASVI) containing 10% (v/v) glycerol. Prior to each experiment, the strain was grown aerobically in tryptic soy agar (TSA, KASVI) at 35º C for 24 h.

2.2 Biofilm formation of E. faecalis V583

The biofilm-forming ability of *E. faecalis* V583 in different conditions (absence and presence of 1%, 5%, 25% and 50% HS) was performed in 96-well cell culture microtiter plates (Greiner CELLSTAR®). In each experiment, the 96-well microplates were filled with tryptic soy broth (TSB, Lab™, Neogen®), 1% D-(+)-glucose (Merck®) and different concentrations of human serum (HS), previously heat-inactivated. Human serum (from male AB plasma, sterile-filtered, H4522) was purchased from Sigma Aldrich®.

The plates were incubated for 8 and 24 h at 37°C under static conditions. Biofilm quantification was performed according protocol from Extremina et al. [12] with 0.2% crystal violet staining (Merck®). The absorbance was measured at 560

nm using GloMax® Discover System (Promega®). Two independent experiments were performed in triplicate.

2.3 Effect of human serum on planktonic growth of E. faecalis V583

A cell suspension of 10^5 cells/ml was prepared in Erlenmeyer flasks with TSBg (TSB plus 1% glucose) and different concentrations of HS (1%, 5%, 25% and 50%). The suspensions were incubated at 35ºC under static conditions. Growth was determined spectrophotometrically by measuring the OD 600nm in different times (0, 2, 4, 6, 12 and 24 h) in order to determine the time-growth curve.

2.4 Expression of virulence genes

In the expression assays, the biofilm induction was performed in six well microplates (Greiner CELLSTAR®), in a final volume of 3 mL per well, added 300 µl of inoculum (1.5 X 10^8 CFU/mL). A control group and 4 different treatments were performed as follows: Control: only TSBg; Treatment 1: TSBg plus 1% HS; Treatment 2: TSBg plus 5% HS; Treatment 3: TSBg plus 25% HS and Treatment 4: TSBg plus 50% HS. All the treatments were performed in triplicate.

The microplates were incubated at 37ºC for 8 hours, in the mid-log phase. After that, the medium was removed for RNA extraction of the planktonic (non-attached) cells and the wells were washed twice with DPBS 1X (Gibco®). One-thousand microliters of deionized water (Sigma Aldrich®) was added in the well, and the sessile (attached) cells were harvested with a cell scraper (240 mm, TPP®). Posteriorly, 500 µl volume of each suspension (planktonic and sessile) was transferred into a sterile microtube of 2mL, followed by vigorous vortexing and sonication to break up cells aggregation. One mL of RNAprotect Bacteria Reagent

(Qiagen®) was added into a microtube for stabilization of RNA prior to RNA isolation procedures. After the RNA stabilization, bacterial cells were pelleted by centrifugation (10 min at 5000 g). The supernatant was decanted and the pellet was frozen at -20ºC until the RNA extraction.

RNA was extracted by enzymatic lysis using lysozyme, and proteinase K digestion followed by purification using the RNeasy Mini Kit (Qiagen®), according manufacturer's instructions. After extraction, the amount of the total RNA extracted was determined using the Thermo Scientific NanoDrop™ 2000 Spectrophotometer, with the 260nm/280nm ratio expected values between 1.8 and 2. Forty ng of high-quality total RNA was reverse-transcribed to complementary DNA (cDNA) in 10 µl volume using the AccuScript High Fidelity RT-PCR System (Agilent Technologies) according the manufacturer's instructions. After that, cDNAs were diluted in the proportion 1:20 and frozen at -20ºC until the performance of the qPCR.

2.5 Reference genes and target genes

Two reference genes and five virulence genes were chosen for the expression assays. The elongation factor Tu, a GTP binding protein involved in peptide chain formation, encoded by *tuf* gene [13] and the 23S Ribossomal RNA, part of the large 50S subunit of the ribosome in prokaryotes, encoded by *23SrRNA* gene [14] were selected as reference genes. Primers for reference genes were obtained using the Primer3Plus software version: 2.4.0 [15]. Primers specificity was evaluated *in silico* with genome of *E. faecalis* V583 (NC_004668.1) using the BLAST® software (National Center for Biotechnology Information). The target genes used in this study were: *E. faecalis* endocarditis antigen encoded by *efaA* gene [4], endocarditis and biofilm-associated pilus encoded by *epbA* gene [5],

adhesion of collagen encoded by *ace* gene [6], gelatinase encoded by *gelE* gene and aggregation substance encoded by *asa* gene [7].

2.6 Quantitative real-time PCR

Quantitative real-time PCR in the StepOnePlus™ Real-Time PCR System (Applied Biosystems) was performed. Master-mix for each PCR run was prepared in 20 µl volume as follows: 10 µl SYBR® Green PCR Master Mix (Applied Biosystems), 5.6 µl water (Sigma-Aldrich®), 4 µl of cDNA and 0.2 µl (0,1 µM) of each primer (sequences in Supplementary table 1). The following amplification program was used: 10 min of denaturation at 95ºC, 40 cycles consisting of 15 s at 95ºC for denaturation, 15 s at (56ºC to *tuf, efaA, ebp, gelE* and *ace* genes; 58ºC to *asa* gene and 60ºC for *23SrRNA* gene) and 45 s at 60ºC for annealing. All reactions were performed in triplicate with three biological replicates.

2.7 Data analysis

Relative expression analysis of each target gene and reference gene in the different treatments was performed in relation to the non-treated samples, through of the Rest 2009 software (M. Pfall and Qiagen) [16] using the crossing points (CP) of each gene studied. Statistical analyses were performed by GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

3. Results

3.1 Effects of human serum supplementation on biofilm formation and planktonic growth

The biofilm-forming ability of *Enterococcus* was evaluated in the absence and presence of 1%, 5%, 25% and 50% of HS after 8 and 24 hours of incubation. At 8 hours, the group supplemented with 5% HS exhibited a decrease of 67% in the biofilm density, compared to the control group. However, no significant differences were observed at 24 hours, when a similar biofilm density was achieved in all conditions (Table 1). The effect of HS on the planktonic growth was evaluated by time growth curve. Compared to the control group (without HS), the presence of different concentrations of HS (1%, 5%, 25% and 50%) did not affect the growth of *E. faecalis* V583 (Fig. 1).

3.3 Effects of human serum supplementation on the expression of the virulence genes ebpA, efaA, ace, gelE and asa

The relative expression of virulence genes was obtained using qPCR. Expression of each gene was evaluated to *E. faecalis* V583 after biofilm induction with 1%, 5%, 25% and 50% of HS. Both sessile and planktonic cells were evaluated in each condition (Fig. 2; Table 1). Among planktonic cells, it was observed a significant upregulation of the *asa* gene in the presence of 5%, 25% and 50% of HS. For other genes, however, no significant differences were observed compared to the control group. In contrast, among sessile cells it was observed a significant upregulation of all genes in high concentrations of HS (25 and 50%). Comparing the gene expression of planktonic cells and sessile cells,

151 *ebpA, efaA, ace* and *gelE* genes were upregulated only in biofilm formation, while
152 *asa* gene showed upregulation in both planktonic and sessile cells.
153
154 **4. Discussion**
155
156 This study evaluated the *in vitro* effects of HS on biofilm formation of a
157 reference strain (*E. faecalis* V583), which were recovered from bloodstream in
158 1987 and is commonly used in different studies of virulence and resistance of
159 enterococci. The process of biofilm formation is complex and involves different
160 steps, being the attachment step critical and dependent on different factors [17,18].
161 In this study, the group of bacteria supplemented with 5% HS, after 8 hours
162 of incubation, produced 67% less biofilm than non-supplemented strain,
163 demonstrating a certain inhibition from HS during the attachment step. Similar
164 results were observed in experiments with *Staphylococcus aureus*,
165 *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* [19–21]. In contrast,
166 Thompson et al. [21] reported an increased biofilm formation capability in the
167 presence of 5% HS supplementation in *S. aureus*. Recently, it was reported that
168 HS in association with N-acetylcysteine also increases biofilm formation in *E.*
169 *faecalis* and other bacteria [22].
170 In our experiments, the effect of HS in biofilm formation was fully reversed
171 after 24 hours of incubation. On the other hand, the planktonic growth had no
172 influence of the supplement.
173 HS presents a complex composition rich in proteins and other nutrients [10],
174 being commonly used as growth factor. Therefore, some components in HS can
175 promote or inhibit biofilm formation. Apo-transferrin, for example, was reported as

a component of HS that could reduce the biofilm formation without affecting the planktonic cell growth of *S. epidermidis* [19,23]. Abraham and Jefferson [8] characterized the inhibitory components of HS in biofilm formation of *S. aureus* and they found a low density protease-resistant that influences the transcription of biofilm-related genes. On the other hand, another study also performed the characterization of the inhibitory component of the HS, and they proposed that a non-proteic component may inhibit the adhesion and biofilm formation of fungi and bacteria [10].

Besides the environmental conditions, the type of surface may also affect the biofilm formation. A study evaluated the influence of the culture medium supplemented with 10% HS on the adhesion of *E. faecalis* ATCC 29212 to glass and silicone rubber, and it was observed that addition of serum did not affect interaction between bacteria and the two surfaces [24].

This study also investigated the direct influence of HS in the biofilm-related genes expression. Planktonic cells are free floating in the medium, while sessile cells are part of the biofilm structure [25]. It is known that there is a physiological difference between these two cells states and that biofilm production is regulated by quorum sensing systems [3,26]. When we compared the transcripts level of each gene among treatments and between cell states, it was observed differences compared to the control group. Sessile cells had more genes regulated than planktonic cells. To understand the interaction of *E. faecalis* with serum as a triggering factor of biofilm formation is important, once they can form biofilms on medical devices and therefore come into contact with blood.

The biofilm formation in *E. faecalis* involves transcriptional regulatory systems or cell-surface components [27]. Many studies prove that *epbA, efaA, ace,*

201 *gelE* and *asa* genes are strongly associated with pathogenesis of *Enterococcus*
202 and also play an individual role in the biofilm biogenesis [28]. In our study, with the
203 exception of the *asa* gene, *epbA, efaA, ace* and *gelE* were upregulated only in
204 sessile cells and in high concentrations of HS. It is known that these genes are
205 influenced by the presence of serum [4,29–31], however our study showed a higher
206 increase of the expression in the sessile cells than planktonic cells, which may be
207 directly associated with the biofilm formation process.

208 Although HS inhibited the biofilm formation of *E. faecalis* at the
209 concentration 5%, our hypothesis is that this concentration of HS was not enough
210 to produce some effect in the gene expression. In the literature, there is no
211 consensus about the optimal concentrations of supplements that promote or inhibit
212 the biofilm formation in different species. For instance, several studies observed
213 regulations in gene expression and biofilm formation effects in a range of HS
214 varying between 3% until 50% [8,10,21].

215 In conclusion, this study demonstrated an *in vitro* inhibitory effect on biofilm
216 formation among *E. faecalis* in low concentration of HS and a significant increase
217 of the biofilm-related gene expression under high concentration of HS. A better
218 understanding of this process as well as the application of these findings *in vivo*,
219 may help in the search for strategies for prevent biofilm formation by this
220 microorganism.

221

222 **Acknowledgements**

223 This study was supported by the Brazilian National Council for Scientific and
224 Technological Development (CNPq) and the Coordination for the Improvement of
225 Higher Education Personnel (CAPES).

Disclosure

There are no conflicts of interest to declare.

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Supplementary Table 1. Primers used in this study:

	Gene	Sequence	Strand	Reference
Translation Elongation Factor Tu [a]	*tuf*	ATTAATGGCTGCAGTTGACG	forward	This study
		AGCAACAGTACCACGTCCAG	reverse	
23S Ribossomal RNA [a]	*23SrRNA*	CAGTGTCAGATGGGCAGTTT	forward	This study
		GCTCCCTTCTGCCTTTACAC	reverse	
Enterococcus faecalis endocarditis antigen [b]	*efaA*	TGGGACAGACCCTCACGAATA	forward	Lowe et al. (1995)
		CGCCTGTTTCTAAGTTCAAGCC	reverse	
Endocarditis and biofilm-associated pilus [b]	*epbA*	AAAAATGATTCGGCTCCAGAA	forward	Bourgogne et al. (2007)
		TGCCAGATTCGCTCTCAAAG	reverse	
Adhesin of collagen [b]	*ace*	GGAGAGTCAAATCAAGTACGTTGGTT	forward	Nallaparedy and Murray. (2006)
		TGTTGACCACTTCCTTGTCGAT	reverse	
		GCCACTTTATCAGCCTGAACC	reverse	
Gelatinase [b]	*gelE*	TATGACAATGCTTTTTGGGAT	forward	Vankerckhoven et al. (2004)
		AGATGCACCCGAAATAATATA	reverse	
Aggregation substance[b]	*asa*	GCACGCTATTACGAACTATATGA	forward	Vankerckhoven et al. (2004)
		TAAGAAAGAACATCACCACGA	reverse	

[a] Reference gene; [b] Target gene

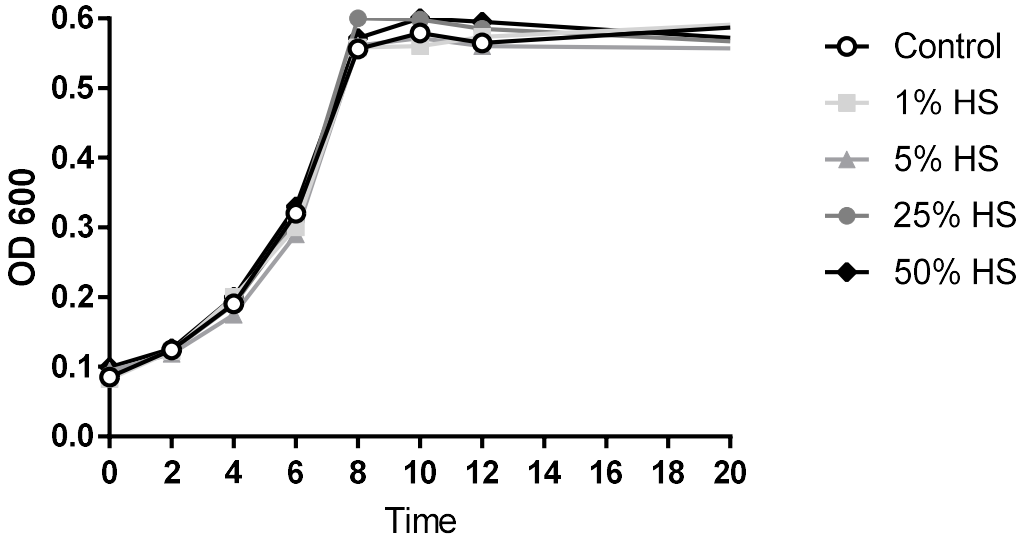

Fig. 1. Effect of human serum on planktonic growth of *Enterococcus faecalis* V583. Control (Tryptic Soy Broth Plus 1% glucose - TSBg), 1% HS (TSBg plus 1% Human Serum), 5% HS (TSBg plus 5% Human Serum), 25% HS (TSBg plus 25% Human Serum) and 50% HS (TSBg plus 50% Human Serum).

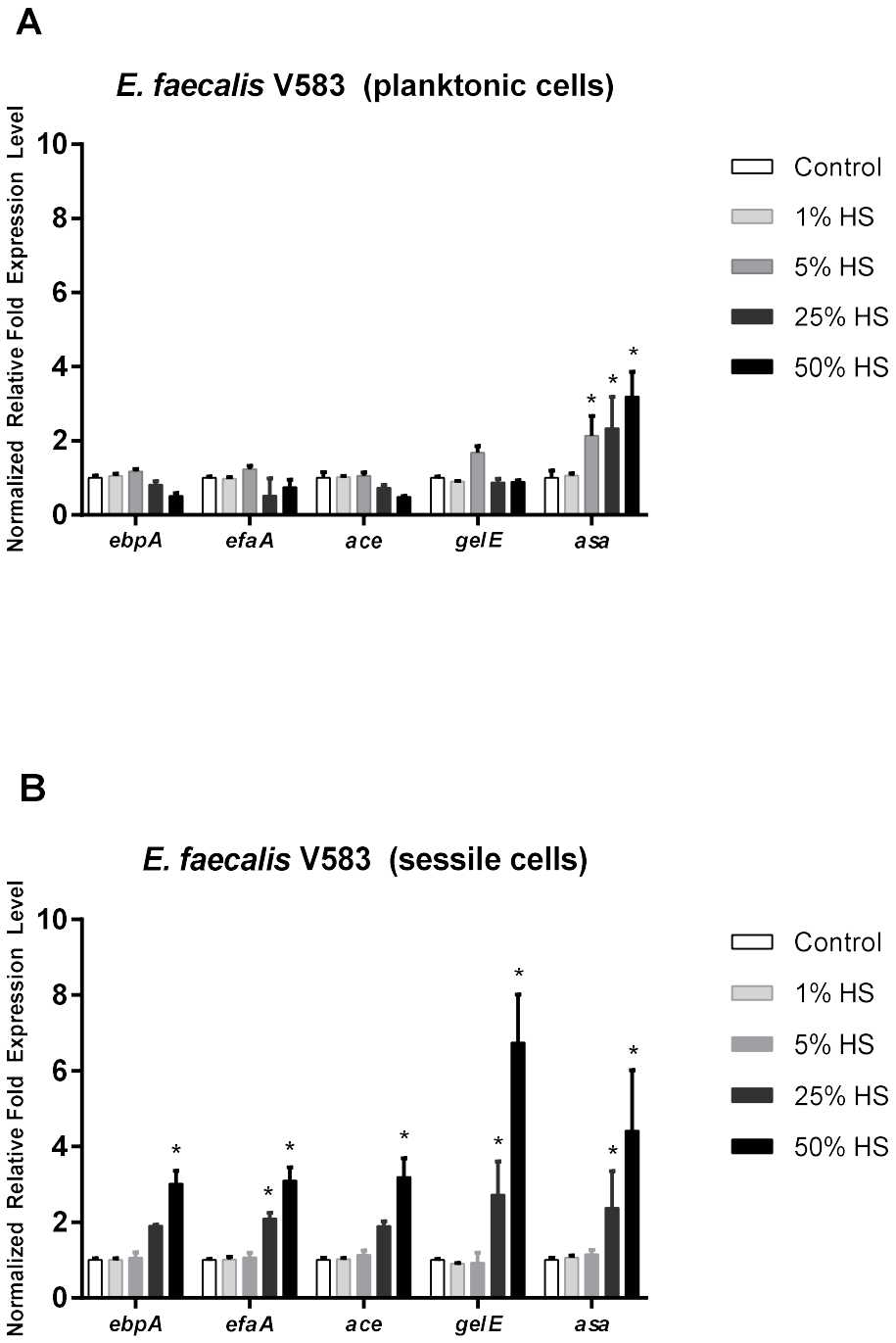

Fig. 2. Expression of *E. faecalis* V583 biofilm-related genes by qPCR. Control (Tryptic Soy Broth Plus 1% glucose - TSBg), 1% HS (TSBg plus 1% Human Serum), 5% HS (TSBg plus 5% Human Serum), 25% HS (TSBg plus 25% Human Serum) and 50% HS (TSBg plus 50% Human Serum). Housekeeping genes *tuf* and *23SrRNA* were used as control. A. Expression of planktonic cells of *E. faecalis* V583. B. Expression of sessile cells of *E. faecalis* V583. Error bars represent standard deviation. Statically differences obtained when compared with control (∗ $P < 0.05$).

Table 1. Gene expression rate and optical density after human serum supplementation (Gene expression assay and biofilm induction performed at 8 h). The values are means ± standard deviations.

		ebpA	*efaA*	*ace*	*gelE*	*asa*	OD** 8 hours	OD** 24 hours
Planktonic	**Control**	1.0 ± 0.06	1.0 ± 0.04	1.0 ± 0.15	1.0 ± 0.04	1.0 ± 0.19	0.556 ± 0.08	0.598 ± 0.06
	1%HS	1.05 ± 0.06	0.98 ± 0.04	1.02 ± 0.03	0.9 ± 0.02	1.06 ± 0.06	0.548 ± 0.04	0.600 ± 0.08
	5%Hs	1.17 ± 0.08	1.23 ± 0.09	1.05 ± 0.1	1.68 ± 0.18	2.13 ± 0.54 *	0.561 ± 0.04	0.556 ± 0.06
	25%HS	0.82 ± 0.09	0.52 ± 0.05	0.74 ± 0.08	0.88 ± 0.1	2.34 ± 0.85 *	0.630 ± 0.07	0.585 ± 0.07
	50%HS	0.51 ± 0.08	0.75 ± 0.21	0.49 ± 0.02	0.9 ± 0.05	3.2 ± 0.67 *	0.572 ± 0.03	0.561 ± 0.07
Sessile	**Control**	1.0 ± 0.05	1.0 ± 0.03	1.0 ± 0.06	1.0 ± 0.3	1.0 ± 0.06	0.811 ± 0.05	1.144 ± 0.09
	1%HS	0.98 ± 0.03	1.01 ± 0.08	1.05 ± 0.03	0.87 ± 0.08	1.12 ± 0.04	0.761 ± 0.08	1.296 ± 0.12
	5%Hs	1.07 ± 0.14	1.07 ± 0.12	1.14 ± 0.12	0.93 ± 0.26	1.16 ± 0.11	0.260 ± 0.09	1.137 ± 0.10
	25%HS	1.92 ± 0.02	2.1 ± 0.15 *	1.91 ± 0.12	2.73 ± 0.89 *	2.34 ± 0.48 *	0.808 ± 0.04	1.105 ± 0.15
	50%HS	3.02 ± 0.34 *	3.11 ± 0.35 *	3.19 ± 0.5 *	6.74 ± 1.2 *	4.42 ± 1.6 *	0.874 ± 0.13	1.127 ± 0.12

* Significant; **Optical density, OD600 to planktonic growth and OD560 to biofilm absorbance.

CHAPTER 5. GENERAL DISCUSSION

With regard to the results already published in the article "**Evaluation of a selective chromogenic medium for detecting vancomycin-resistant enterococci",** the correct identification of Vancomycin Resistant Enterococci (VRE) in surveillance culture is important once that colonization and infection with VRE is an increasing problem worldwide and its associated with the risk of developing Healthcare Associated Infections (HAI) (FLOKAS et al., 2017; ÖZSOY & İLKI, 2017). Screening for VRE is a routine procedure, and the use of good tools can improve the diagnosis, thus avoiding the spread of this pathogen and the incorrect prescription of antibiotics. In this research, we evaluated the performance of a selective chromogenic medium for the detection and differentiation of vancomycin-susceptible and vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. The chromID™ VRE agar had a high sensitivity, similar to previous studies evaluating the performance of the same medium (DELMAS et al., 2007; CUZON et al., 2008; GRABSCH et al., 2008; ASIR et al., 2009). In contrast, the specificity of the method was significantly lower compared to the same studies. Besides, we observed colonies with non-discriminatory staining that may lead to false-negative results. Therefore, we recommended the chromID™ VRE, once it is a rapid and useful tool for the screening and identification of VRE, as long as it is associated with conventional tests.

Concerning manuscript on "**High diversity of Vancomycin-resistant *Enterococcus faecium* isolated in Southern Brazil**", VRE_{fm} has become one of the leading causes of HAI, such as bacteremia and infective endocarditis (O'DRISCOLL & CRANK, 2015; TEDIM et al., 2017), and responsible for hospital outbreaks worldwide (WILLEMS et al., 2005). In this context, we described the susceptibility profile and the

clonal relationship of 115 VRE_{fm} consecutively recovered from inpatients of three general hospitals in Southern Brazil, from September/2012 to April/2017. All *E. faecium* exhibited resistance to vancomycin (harboring *vanA* gene), teicoplanin, ampicillin and ciprofloxacin, and 13.9% high-level resistance to gentamicin, showing a multidrug-resistant profile. Most part of VRE_{fm} isolates harbored *esp* and *acm* genes, both related to biofilm formation and adherence to extracellular matrix (HENDRICKX et al., 2007). Some of these characteristics found in our isolates are similar to the CC-17 lineage, a well-adapted lineage to the hospital environment and associated with most of the reported hospital outbreaks worldwide (WILLEMS et al., 2005; PANESSO et al., 2010). The VRE_{fm} isolates were separated by PFGE into 38 types (23 clonal types and 15 *singletons*), showing a polyclonal distribution of VRE_{fm} in Southern Brazil, also observed in other studies (POURSHAFIE et al., 2008; TOP et al., 2008a; LANDERSLEV et al., 2016; SOMILY et al., 2016).

In the manuscript **"Influence of glucose supplementation on biofilm formation and expression of associated-biofilm genes in *Enterococcus faecalis* clinical isolates**", we aimed to assess the *in vitro* effects of glucose on biofilm formation and gene expression of *Enterococcus faecalis* clinical isolates. The presence of glucose seems to be an essential factor for biofilm formation by different bacteria, as well, contributes to bacterial growth, reproduction and metabolic activities (PILLAI et al., 2004; MARINHO et al., 2013; SENEVIRATNE et al., 2013; RAN et al., 2015). The biofilm induction was performed in the presence and absence of glucose, and the 1% glucose supplementation significantly increased biofilm formation in all isolates evaluated already at 8 hours of induction. The direct influence of glucose in the expression of the biofilm-related genes was also investigated to eleven *E. faecalis* clinical isolates and one reference strain (*E. faecalis* ATCC 29212), possessing all the

four genes (*ebpA, efaA, ace* and *gelE*). PILLAI et al. (2004) showed that 1% glucose supplementation increased the optical density and can regulate, directly or indirectly, the transcription of some glucose-dependent genes associated with biofilm formation in *E. faecalis*. In our study, all four biofilm-associated genes were up-regulated in 1% glucose in a subgroup of isolates and no significant differences were observed for the others. Interestingly, our findings demonstrated that some genes had not its expression altered in the presence of glucose during the biofilm formation, showing that patterns of virulence gene expression are dependent on the bacterial isolate.

Finally, in the manuscript **"Human serum enhances virulence factors expression and inhibits adhesion of *E. faecalis* V583"**, we evaluated the *in vitro* effects of human serum on biofilm formation of a reference strain (*E. faecalis* V583). Human serum presents a complex composition rich in proteins and other nutrients (DING et al., 2014), is commonly used as a growth factor. Some of these components (protein or non-protein) may promote or inhibit biofilm formation (ARDEHALI et al., 2003; ABRAHAM & JEFFERSON, 2010; DING et al., 2014; SHE et al., 2016). In our study, the group supplemented with 5% human serum, at 8 hours incubation, produces 67% less biofilm than non-supplemented strain, demonstrating a certain inhibition from human serum during the attachment step, also observed in experiments performed with *Staphylococcus aureus* and *P. aeruginosa* (ABRAHAM; JEFFERSON, 2010; HAMMOND et al., 2010; SHE et al., 2016). No influence of human serum was observed in the planktonic growth of *E. faecalis* V583. Our study also investigated the direct influence of human serum in the expression of the biofilm-related genes, in planktonic and sessile cells. It is known that there is a physiological difference between these two cells states (MOHAMED; HUANG, 2007; STEWART & FRANKLIN, 2008), and when we

compared the transcripts level of each gene among treatments and between cell states, it was observed differences in relation to the control group. Sessile cells had more genes regulated than planktonic cells, which may be directly associated with the biofilm formation process. Understand the interaction of *E. faecalis* with serum as a triggering factor of biofilm formation is important, once they can form biofilms on medical devices and therefore come into contact with blood.

CHAPTER 6. CONCLUSION

Responding to the specific aims of this thesis, we evaluated the performance of ChromID VRE® in the identification of 184 well-characterized *Enterococcus* spp. and reference strains. ChromID VRE® is a commercial agar to detect VRE, commonly used in routine laboratories, and in our experiments had a good sensitivity of 95.52% but a low specificity of 30% due to false positive results. We recommend further VRE identification in order to avoid misinterpretation and unnecessary antimicrobial therapy.

We verify the multidrug resistance profile of all 115 vancomycin resistance enterococci clinical isolates, similar to the VRE profile spread around the world. Besides vancomycin, all the VRE strains were resistant to ampicillin, ciprofloxacin, and teicoplanin. Some of these were also resistant to high levels of gentamicin. However, all the VRE strains were susceptible to linezolid, daptomycin, and quinupristin-dalfoprisitin, the therapeutic options to treat multidrug-resistant enterococcal infection, showing that so far, the resistance to these antimicrobials did not spread yet in Southern Brazil.

We verified that all VRE isolates harbored *vanA* gene, that encodes resistance to high levels of vancomycin and resistance to teicoplanin. The *vanB* gene was not present in neither of the VRE strains. Besides, a high prevalence of the virulence genes *esp* and *acm* was observed among them, being present in 86.1% and 95.7%, respectively. Resistance to vancomycin, ampicillin and ciprofloxacin and presence of *vanA* and *esp* genes are features of a genetic lineage of *E. faecium* designated as clonal complex-17, associated with most of the reported hospital outbreaks worldwide.

We performed the molecular characterization of the 115 VRE isolates by PFGE and we verified a high clonal diversity among them. Seventy-nine isolates were grouped in 23 clonal types and 15 isolates exhibited unique pattern type. Twenty-one

isolates were characterized as non-typable because no band pattern was generated after macrorestriction with *SmaI* enzyme, showing a limitation of the PFGE technique.

We verified the presence of the biofilm-associated genes *ebpA, efaA, ace* and *gelE* in 123 *E. faecalis* previously characterized as biofilm formers. Forty-two (34.1%) isolates harbored the four genes. The *efaA* was the most prevalent gene, present in 95.9% of the *E. faecalis* isolates, followed by, *gelE* gene (70.7%), *ebpA* gene (69.1%) and *ace* gene (64.2%).

We evaluated the *in vitro* biofilm formation in the absence and presence of glucose supplementation in 123 *E. faecalis* previously characterized as biofilm formers, and we verified that 1% glucose increases significantly the biofilm capability. In view of this, we also verify the influence of 1% glucose in the expression of biofilm-associated genes in 11 isolates and one control, whose harbored all the four genes and were affected by the presence of glucose. We verified that the gene expression varied among the isolates, showing that patterns of virulence gene expression are not the same to all isolates from the same species.

Finally, we evaluated the *in vitro* influence of different concentrations of human serum (1%, 5%, 25% and 50%) in the biofilm formation and virulence gene expression (*ebpA, efaA, ace, gelE* and *asa*) of *E. faecalis* V583 (ATCC 700802), and we verified and inhibition of 5% human serum in the initial step of biofilm biogenesis, however, this concentration did not affect the gene expression. On the other hand, high concentrations of human serum (25% and 50%) increased the expression of *ebpA, efaA, ace* and *gelE* genes in sessile cells and the expression of *asa* gene in both planktonic and sessile cells, showing a directly influence of the human serum in the expression of the virulence genes during the biofilm formation.

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Ethics committee approval of UFCSPA

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Epidemiologia Molecular e Análise de Expressão de Genes de Resistência e Virulência em Enterococcus isolados a partir de amostras clínicas em Porto Alegre, RS

Pesquisador: Pedro Alves d'Azevedo
Área Temática:
Versão: 2
CAAE: 47197915.2.0000.5345
Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre
Patrocinador Principal: CNPQ

DADOS DO PARECER

Número do Parecer: 1.283.544

Apresentação do Projeto:
Enterococcus faecalis e Enterococcus faecium estão entre os principais agentes etiológicos de infecções associadas à assistência à saúde (IAAS). Fatores de virulência estão associados com o processo de invasão, avanço e proliferação no hospedeiro, desempenhando um papel crucial na persistência desse patógeno nos mais diversos sítios anatômicos. O surgimento da resistência adquirida a antibióticos sistêmicos que antes eram eficazes no tratamento de infecções enterocócicas também se tornaram uma preocupação crescente, principalmente ao antimicrobiano vancomicina considerada a opção terapêutica para tratamento de Enterococcus resistentes aos antimicrobianos de escolha primária. O isolamento de Enterococcus resistentes à vancomicina (VRE) tem se tornado cada vez mais comum e relatado em diferentes regiões do mundo, o que está frequentemente associado com um padrão de multirresistência que dificulta o tratamento de infecções por estes micro organismos. Neste contexto, o presente estudo tem como foco a caracterização da distribuição das espécies de VRE pertencentes à bacterioteca do laboratório de cocos gram-positivos da UFCSPA obtidas de projetos anteriores, isolados a partir de diferentes amostras clínicas e de culturas de vigilância, avaliar o perfil de suscetibilidade dos isolados aos antimicrobianos comumente utilizados no tratamento, detectar a presença e expressão dos genes associados à virulência e à resistência e caracterizar molecularmente os isolados pelos métodos de

Endereço: Rua Sarmento Leite ,245
Bairro: Sarmento **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3303-8804 **E-mail:** cep@ufcspa.edu.br

genotipagem PFGE e MLST.

Objetivo da Pesquisa:
Objetivo Primário: Avaliar epidemiologicamente e genotipicamente isolados clínicos de Enterococcus isolados a partir de amostras clínicas em Porto Alegre, RS.

Objetivo Secundário:
- Avaliar o perfil de suscetibilidade dos isolados de ERV aos antimicrobianos comumente utilizados no tratamento, através dos métodos de detecção da concentração inibitória mínima (CIM).
- Realizar a Caracterização Molecular dos isolados de ERV pelos métodos de genotipagem PFGE e MLST.
- Avaliar a capacidade de formação de biofilme.
- Avaliar a expressão de genes de resistência e virulência e a produção de moléculas sinalizadoras do sistema quorum sensing.

Avaliação dos Riscos e Benefícios:
Riscos: Este estudo não utiliza pacientes nem dados clínicos dos mesmos, serão utilizados na pesquisa exclusivamente isolados bacterianos. Em nenhuma das etapas do projeto será empregado material biológico humano (espécimes, amostras e alíquotas e material original e seus componentes fracionados), conforme descrito na Resolução nº441, de 12 de maio de 2011, do Conselho Nacional de Saúde. Portanto, os riscos apresentados são os do manuseio dos isolados bacterianos por parte do pesquisador, para o qual será minimizado pela utilização de EPIs.

Benefícios: Os benefícios obtidos neste estudo são unicamente científicos, e estão associados a compreensão da epidemiologia dos Enterococcus resistentes à vancomicina isolados na cidade de Porto Alegre, bem como se há alguma relação genética entre os isolados que possa estar relacionada com a dispersão desse micro-organismo no ambiente hospitalar, podendo auxiliar na prevenção e controle da sua disseminação.

Comentários e Considerações sobre a Pesquisa:
Projeto bem estruturado e fundamentado. Apresenta potencial de contribuição científica para a área.

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Considerações sobre os Termos de apresentação obrigatória:

Propõe dispensa do TCLE uma vez que não haverá contato com pacientes e nem utilização ou contato direto com amostras clínicas de pacientes. Só serão analisadas neste estudo isolados bacterianos que encontram-se na bacterioteca do Laboratório de Cocos-Gram positivos da UFCSPA.

Recomendações:

Pendência atendidas. Aprovar.

Conclusões ou Pendências e Lista de Inadequações:

Aprovar.

Considerações Finais a critério do CEP:

Termino: Dez/2017

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_487858.pdf	16/09/2015 18:41:40		Aceito
Outros	Termo_anuencia.pdf	16/09/2015 18:41:13	Renata Oliveira Soares	Aceito
Outros	Termo_Compromisso.pdf	16/09/2015 18:40:30	Renata Oliveira Soares	Aceito
Projeto Detalhado / Brochura Investigador	Projeto Enterococcus - CEP 2015.pdf	11/07/2015 10:43:18		Aceito
Folha de Rosto	Folha de Rosto.pdf	07/07/2015 10:23:13		Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Endereço: Rua Sarmento Leite ,245
Bairro: Sarmento **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3303-8804 **E-mail:** cep@ufcspa.edu.br

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Continuação do Parecer: 1.283.544

PORTO ALEGRE, 16 de Outubro de 2015

Assinado por:
Julia Fernanda Semmelmann Pereira Lima
(Coordenador)

Endereço: Rua Sarmento Leite ,245
Bairro: Sarmento **CEP:** 90.050-170
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3303-8804 **E-mail:** cep@ufcspa.edu.br

Publications

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4. PAIM, THIAGO G.S.; PIETA, LUIZA; PRICHULA, JANIRA; SAMBRANO, GUSTAVO E.; SOARES, RENATA; BELLO, ALINE DALL; FRAZZON, JEVERSON; D'AZEVEDO, PEDRO A. **Draft Genome Sequence of Strain F165 Isolated from a Urinary Tract Infection.** Genome Announcements. 2016; 4:e01084-16.

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Congress Abstracts

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2. SOARES, R.O.; RAMOS, F.S.; PRICHULA, JANIRA; ROSSATO, A.M.; d'AZEVEDO, P.A. **Effects of human serum on biofilm formation of *Enterococcus faecalis* clinical isolates.** In: 5th International Conference on Enterococci, 2018, Chamonix, France.

3. CANANI, C.R.; SOARES, R.O.; COSTA, L.F.X.; GRASSOTTI, T.T.; FRAZZON, A.P.G. **Análise do perfil de suscetibilidade a antimicrobianos e detecção de genes de resistência em *Enterococcus faecalis* isolados de infecções urinárias.** In: Simpósio Brasileiro de Microbiologia Aplicada X Simpósio Brasileiro de Microbiologia Aplicada / IV Encontro Latino-Americano de Microbiologia Aplicada, 2017, Porto Alegre, Brazil.

4. RAUBER, J.M.; BAGGIOTTO, B.; ZANOTTO, M.B.; CARNEIRO, M.; SOARES, R.O.; VALIM, A.R.M.; d'AZEVEDO, P. A. **The importance of therapeutic drug monitoring of vancomycin in cases of empirical treatment and staphylococcal infections**. In: 26th European Congress of Clinical Microbiology and Infectious Disease, 2016, Amsterdam, Netherlands.

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6. SOARES, R. O.; ROSSATO, A.M.; TOLFO, N.C.C.; PAIM, T.G.S.; SAMBRANO, G.E.; SANTIN, J.T.; KISWESKI, A.E.; d'AZEVEDO, P.A. **Antimicrobial susceptibility profile and molecular characteristics of *Staphylococcus aureus* colonizing patients with epidermolysis bullosa.** In: 26th European Congress of Clinical Microbiology and Infectious Disease, 2016, Amsterdam, Netherlands.

7. MARTINS, N.D.; ROSSATO, A.M.; SOARES, R.O.; SAMBRANO, G.E.; PAIM, T.G.S.; d'AZEVEDO, P.A. **Detecção de genes de virulência em *Staphylococcus aureus* resistente à meticilina.** In: IX Simpósio Brasileiro de Microbiologia Aplicada, 2016, Porto Alegre, Brazil.

8. ROSSATO, A.M.; MARTINS, N.D.; SOARES, R.O.; SAMBRANO, G.E.; PAIM, T.G.S.; d'AZEVEDO, P.A. **Tipagem molecular de *Staphylococcus aureus* resistente à meticilina com reduzida susceptibilidade à vancomicina.** In: IX Simpósio Brasileiro de Microbiologia Aplicada, 2016, Porto Alegre, Brazil.

9. PAIM, T.G.S.; SAMBRANO, G.E.; SOARES, R.O.; MOURA, T.M.; d'AZEVEDO, P.A. **Antibacterial activity by time-kill assay of linezolid in combination of antimicrobial drugs against Vancomicin-Resistant *Enterococcus faecium* (VREfm) isolate.** In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis, Brazil.

10. SOARES, R. O.; TOLFO, N.C.C.; PAIM, T.G.S.; SAMBRANO, G.E.; CAIERAO, J.; d'AZEVEDO, P.A. **Evaluation of a selective chromogenic medium for the detection of Vancomycin Resistant Enterococci previously characterized for phenotypic and molecular methods.** In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis, Brazil.

11. SAMBRANO, G.E.; PAIM, T.G.S.; SOARES, R.O.; TOLFO, N.C.C.; THORN, C.; d'AZEVEDO, P. A. **Susceptibility profile of Streptococcus pyogenes isolated from patients in two hospitals in porto alegre, Rio Grande do Sul, Brazil.** In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis, Brazil.

12. SANTOS, J.N.; SOARES, R.O.; BEGUET, K.; d'AZEVEDO, P.A.; PEREZ, V. P. **Antimicrobial susceptibility of Vancomycin Resistant Enterococci (VRE) recovered from surveillance culture of patients admitted to a hospital in the metropolitan region of Porto Alegre-RS.** In: 28º Congresso Brasileiro de Microbiologia, 2015, Florianópolis, Brazil.

13. ROSSATO, A.M.; SAMBRANO, G.E.; REITER, K.; SOARES, R.O.; PAIM, T.G.S.; d'AZEVEDO, P.A. **Perfil de susceptibilidade aos antimicrobianos de *Staphylococcus aureus* resistentes à meticilina (MRSA) isolados a partir de origens clínicas diversas, em hospitais de Porto Alegre, RS, Brasil**. In: VIII Simpósio Brasileiro de Microbiologia Aplicada, 2015, Porto Alegre, Brazil.

Instructions for publication

1. **Journal of Infection, Genetics and Evolution:** Guide for authors available in https://www.elsevier.com/journals/infection-genetics-and-evolution/1567-1348/guide-for-authors

2. **Journal of Microbial Pathogenesis:** Guide for authors available in https://www.elsevier.com/journals/microbial-pathogenesis/0882-4010/guide-for-authors

3. **Journal of Research in Microbiology:** Guide for authors available in https://www.elsevier.com/journals/research-in-microbiology/0923-2508/guide-for-authors