Presence of virulence factors in *Enterococcus faecalis* and *Enterococcus faecium* susceptible and resistant to vancomycin

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Despite the increasing importance of Enterococcus *as opportunistic pathogens, their virulence factors are still poorly understood. This study determines the frequency of virulence factors in clinical and commensal* Enterococcus *isolates from inpatients in Porto Alegre, Brazil. Fifty* Enterococcus *isolates were analysed and the presence of the* gelE, asa1 *and* esp *genes was determined. Gelatinase activity and biofilm formation were also tested. The clonal relationships among the isolates were evaluated using pulsed-field gel electrophoresis. The* asa1, gelE *and* esp *genes were identified in 38%, 60% and 76% of all isolates, respectively. The first two genes were more prevalent in* Enterococcus faecalis *than in* Enterococcus faecium, *as was biofilm formation, which was associated with* gelE *and* asa1 *genes, but not with the* esp *gene. The presence of* gelE *and the activity of gelatinase were not fully concordant. No relationship was observed among any virulence factors and specific subclones of* E. faecalis *or* E. faecium *resistant to vancomycin. In conclusion,* E. faecalis *and* E. faecium *isolates showed significantly different patterns of virulence determinants. Neither the source of isolation nor the clonal relationship or vancomycin resistance influenced their distribution.*

Key words: *Enterococcus* - virulence factors - vancomycin resistance

In recent decades, *Enterococcus* have emerged as important nosocomial pathogens. Currently, they are recognised as the second-most common cause of urinary tract infections and the third-most common cause of nosocomial bacteraemia (Lindenstrau et al. 2011). Although many species can be recovered from human infections, *Enterococcus faecalis* is the most frequently recovered, representing more than 90% of clinical isolates, followed by *Enterococcus faecium* (Dahlén et al. 2012), which has shown an increase in prevalence in recent years (Fisher & Phillips 2009). Classically, *E. faecalis* has been linked to increased virulence, whereas *E. faecium* commonly exhibits multiresistance characteristics (Sharifi et al. 2012).

Recent studies have associated enterococcal virulence with different factors, such as gelatinase production, *Enterococcus* surface protein (Esp), aggregation substance (AS) and biofilm formation (Chuang et al. 2009). Gelatinase is a zinc metalloprotease, encoded by *gelE*, with hydrolytic capacity (Lindenstrau et al. 2011). AS, encoded by a plasmid gene, mediates binding to the host epithelium and it appears to mediate bacterial aggregation during conjugation, facilitating plasmid exchange (Schlievert et al. 2010). The Esp protein, encoded by the *esp* gene, seems to contribute to the colonisation and persistence of *E. faecalis* strains in ascending infections of the urinary tract. In addition, Esp may mediate the interaction with primary surfaces and participate in biofilm formation (Chuang-Smith et al. 2010), which substantially enhances bacterial survival in biopolymers and may also be involved in antimicrobial resistance (Ballering et al. 2009).

Knowledge of the virulence characteristics of circulating *Enterococcus* strains may help to understand the complex pathogenic process of these opportunistic microorganisms (Sharifi et al. 2012). Data about virulence of *Enterococcus* circulating strains in Brazil are still scarce (Ruzon et al. 2010). Therefore, the objective of this study was to evaluate the presence of virulence factors in *E. faecalis* and *E. faecium* from clinical and surveillance samples.

SUBJECTS, MATERIALS AND METHODS

Enterococcus were isolated from inpatients in Porto Alegre, southern Brazil, between 2000-2011. The bacteria were recovered from two distinct situations: an *E. faecalis* outbreak that occurred mainly during 2004, with some related strains isolated from 2000 until 2009, and an *E. faecium* outbreak that occurred during 2010-2011. To potentially increase the genetic diversity of the strains, non-epidemiologically related *Enterococcus* strains recovered during the same period were examined. The isolates were from clinically relevant sites and from surveillance cultures (rectal swabs), which were performed by infection control staff during the first outbreak. The samples were maintained in 10% (v/v) Skim Milk solution (Molico, Nestlé®) with 10% glycerol (v/v) and stored at -20ºC.

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Isolates were identified by conventional biochemical tests (Teixeira et al. 2011) and susceptibility to vancomycin was determined following the Clinical Laboratory Standards Institute guidelines (CLSI 2011) using broth microdilution to determine the minimal inhibitory concentration (MIC).

Gelatinase activity was characterised as described elsewhere (Marra et al. 2007). Briefly, samples were inoculated into tubes containing 4 mL of brain heart infusion broth with 4% gelatin. After incubation at 35-37ºC for 24 h, the tubes were cooled at 4ºC for 30 min and the liquefaction of the medium was observed.

Biofilm formation was determined using the method described proposed by Stepanovic et al. (2000). Briefly, microorganisms were grown in polystyrene microplates and the wells were washed, stained with crystal violet and washed again. The dye was solubilised using an alcohol solution. Then, the absorbance of the solution was measured by determining the optical density (OD) of the wells. The cut-off was defined as the mean OD of eight negative control wells. Based on the OD, the isolates were classified as follows: non-adherent, weakly adherent, moderately adherent and strongly adherent.

The presence of the *gelE*, *esp* and *asa1* genes was verified by polymerase chain reaction, as described by Eaton and Gasson (2001). The sequences of primers and annealing temperatures used are shown in Table I. Statistical analysis was performed with SPSS version 19.0, using the chi-square test. The clonal relationships among the isolates were determined by pulsed-field gel electrophoresis (PFGE) and by comparing the fragment patterns after restriction of chromosomal DNA with SmaI, as described elsewhere (Teixeira et al. 1997). Electrophoresis was performed in a CHEF DR III (Bio-Rad) apparatus.

RESULTS

This study evaluated 50 *Enterococcus* isolates: 30 *E. faecalis* and 20 *E. faecium*. Twenty *E. faecalis* were vancomycin-resistant enterococci (VRE), with a MIC ≥ 256 µg/mL, as were all *E. faecium*. Table II describes the characteristics of the *Enterococcus* included in the study. Most of the isolates were recovered from urine (30%). Other specimens included blood, stool (surveillance cultures), abdominal secretions, catheters, wounds, soft tissue and nephrostomy secretions. Seven isolates had an unidentified source. No relationship was found between the source of isolation and the presence or activity of any virulence factor.

The distribution of virulence factors among the isolates is shown in Table III. Overall, 60% (30/50) of the enterococci carried *gelE*. Of these, 66.6% (20/30) presented gelatinase activity. The *esp* gene was detected in 76% (38/50) of isolates and 38% (19/50) of all *Enterococcus* carried the *asa1* gene. Furthermore, 74% (37/50) of the isolates were biofilm formers. Most *E. faecalis* were moderate or strong formers, whereas most *E. faecium* were not able to produce biofilm, or if so, the biofilm was weakly adherent.

Considering vancomycin resistance as a variable, we did not find any significant difference in the presence or activity of virulence factors between resistant and susceptible enterococci. On the other hand, the presence and activity of virulence factors were significantly different between the species. Only VRE isolates were selected to compare virulence characteristics between *E. faecalis* and *E. faecium*.

E. faecalis carried *gelE* at a significantly higher frequency and presented gelatinase activity more frequently than *E. faecium* ($p < 0.001$). The presence of the *asa1* gene was also significantly different between the two species ($p < 0.001$), being more commonly found in *E. faecalis* than in *E. faecium*. On the other hand, there was no difference in the frequency of the *esp* gene between the two species. Significantly more *E. faecalis* isolates formed biofilms than *E. faecium* isolates ($p = 0.003$).

We also evaluated the association between biofilm formation and the presence of the *esp*, *asa1* and *gelE* genes in *E. faecalis* and *E. faecium*. Biofilm formation was significantly associated with the presence of the *gelE* ($p= 0.001$) and *asa1* genes ($p = 0.03$), although it was not associated with the *esp* gene ($p = 0.06$). Nevertheless, all strongly adherent isolates carried, along with *gelE*, the *esp* gene and more than 70% of the strongly adherent isolates carried all three virulence genes.

PFGE analysis was performed in three groups of isolates: *E. faecalis* resistant to vancomycin (VRE_f) -

TABLE I

Primers and annealing temperatures (AT) used to detect the virulence genes

Gene	Primers	Sequence (5'-3')	Product (bp)	AT (°C)
gelE	TE9	ACCCCGTATCATTGGTTT	419	53
	TE10	ACGCATTGCTTTTCCATC		
esp	TE34	TTGCTAATGCTAGTCCACGACC	933	52
	TE36	GCGTCAACACTTGCATTGCCGAA		
agg	TE3	AAGAAAAAGAAGTAGACCAAC	1,553	52
	TE4	AAACGGCAAGACAAGTAAATA		

bp: base pairs.

TABLE II

Characteristics of *Enterococcus* isolates included in the study

Species	Vancomycin MIC (μg/mL)	Source	Clonal type	Year
E. faecalis	> 256	Stool	F_B	2000
E. faecalis	> 256	Urine (n = 2)	F_{A2}	2001/2004
E. faecalis	> 256	Stool	F_{A2}	2004
E. faecalis	> 256	NI	F_{A1}	2004
E. faecalis	> 256	Urine	F_{A1}	2004
E. faecalis	> 256	Blood (n = 4)	F_{A1}	2004/2004/2005/2008
E. faecalis	> 256	Nefrostomy secretion	F_{A1}	2004
E. faecalis	> 256	Stool (n = 4)	F_{A1}	2004
E. faecalis	> 256	Catheter	F_{A1}	2004
E. faecalis	> 256	Wound	F_{A1}	2004
E. faecalis	> 256	NI	F_{A1}	2006
E. faecalis	> 256	Sputum	F_{A1}	2008
E. faecalis	> 256	Abdominal secretion	F_{A1}	2008
E. faecalis	1	Soft tissue	S_A	2008
E. faecalis	0.5	Blood	S_B	2008
E. faecalis	0.5	Soft tissue	S_C	2009
E. faecalis	1	NI	S_D	2009
E. faecalis	2	Abdominal secretion	S_E	2009
E. faecalis	1	NI	S_F	2009
E. faecalis	0.5	NI	S_G	2010
E. faecalis	0.25	NI	S_H	2010
E. faecalis	2	NI	S_I	2010
E. faecalis	0.5	Abdominal secretion	S_J	2010
E. faecium	> 256	Urine	E_B	2010
E. faecium	> 256	Blood (n = 2)	E_{A2}	2011
E. faecium	> 256	Blood (n = 5)	E_{A1}	2011
E. faecium	> 256	Urine (n = 2)	E_{A2}	2011
E. faecium	> 256	Urine (n = 8)	E_{A1}	2011
E. faecium	> 256	Abdominal secretion	E_{A1}	2011

MIC: minimal inhibitory concentration; NI: not identified.

TABLE III

Distribution of virulence genes, the gelatinase activity and biofilm formation among isolates

Group (n)	Clonal type (n)	*agg* n (%)	*esp* n (%)	*gelE* n (%)	Gelatinase n (%)	Biofilm n (%)	Virulence genes
VRE_{fm} (20)	E_{A1} (15)	1 (5)	13 (65)	-	1 (5)	7 (35)	*agg*, *esp*, gelatinase, biofilm
	E_{A2} (3)	-	2 (10)	1 (5)	-	1 (5)	*esp*, *gelE*, biofilm
	E_B (1)	-	1 (5)	-	-	1 (5)	*esp*, biofilm
	Total	1 (5)[a]	16 (80)	1 (5)[a]	1 (5)[a]	9 (45)[a]	-
VRE_f (20)	F_{A1}	11 (55)	14 (70)	18 (90)	13 (65)	17 (85)	*agg*, *esp*, *gelE*, gelatinase, biofilm
	F_{A2}	2 (10)	1 (5)	-	-	1 (5)	*agg*, *esp*, biofilm
	F_B	-	1 (5)	1 (5)	-	1 (5)	*esp*, *gelE*, biofilm
	Total	13 (65)[a]	16 (80)	19 (95)[a]	13 (65)[a]	19 (95)[a]	-
VSE_f (10)	NRI	5 (50)	6 (60)	10 (100)	6 (60)	9 (90)	*agg*, *esp*, *gelE*, gelatinase, biofilm
Total	Total	19 (38)	38 (76)	30 (60)	20 (40)	37 (74)	-

a: $p < 0.05$; NRI: non-related isolates (unique profiles); VRE_f: *Enterococcus faecalis* resistant to vancomycin; VRE_{fm}: *E. faecium* resistant to vancomycin; VSE_f: *E. faecalis* susceptible to vancomycin.

representative isolates of the first outbreak period), *E. faecium* resistant to vancomycin (VRE_{fm}) (representative isolates of the second outbreak period) and *E. faecalis* susceptible to vancomycin (VSE_f), which was included to possibly increase the genetic variability. Although VRE_f and VRE_{fm} presented a clonal relationship, with a major clone each, the distribution of virulence factors was not homogeneous among the isolates (Table III). The major clones of VRE_f and VRE_{fm} represented 19 of each 20 isolates, with two subclones that were highly related. On the other hand, VSE demonstrated a highly heterogeneous PFGE profile (no clonal relationship among the isolates was observed), with most isolates presenting all evaluated virulence determinants.

DISCUSSION

Studies focused on enterococcal virulence are complex because the essential factors for pathogenicity have not yet been described. Indeed, virulence in this genus has been typically considered a multifactorial process, with the participation of several genes and their products.

Production of gelatinase is a characteristic mediated by the *gelE* gene and phenotypically expressed in vitro by liquefaction of a culture medium containing the substrate (Tsikrikonis et al. 2012). We observed *Enterococcus* isolates that did not produce the enzyme, even though *gelE* was detected. Indeed, according to Marra et al. (2007), the presence of *gelE* is not necessarily correlated with gelatinase activity. Some studies suggest that other genes may be associated with *gelE* expression control. Indeed, Lindenstrau et al. (2011) indicated that mutated genes affect *gelE* gene expression and possibly regulate gelatinase production. These findings reinforce the complexity of the processes involved in *Enterococcus* virulence.

There are diverging opinions concerning the role of Esp in biofilm production (Garth et al. 2008). Dworniczek et al. (2012) concluded that there is no clear relationship between the expression of *esp* or *gelE* and biofilm formation. Indeed, an analysis by Sillanpää et al. (2010) showed efficient biofilm production in the absence of Esp in *E. faecium* isolates. On the other hand, Chuang-Smith et al. (2010) speculated that Esp may mediate the interaction with primary surfaces and participate in the formation of this phenotype. In addition, Heinkens et al. (2007) showed that Esp is involved in biofilm formation in *E. faecium* isolates. As our results did not show an association between the presence of *esp* and biofilm production, we assume that other factors are associated with this phenotype.

We observed a considerable number of *E. faecium* carrying *esp* compared with *asa1* and *gelE* genes, which were detected at very low frequencies among this species. Some clones of vancomycin-resistant *E. faecium* that are well established in hospitals show high positivity for *esp,* suggesting that this gene may play an important role in the virulence process (Willems & Bonten 2007). Besides *esp*, Willems et al. (2005) detected some other genes and combinations of mutations and recombinations that give nosocomial *E. faecium* clones adaptive advantages that contribute to their dissemination.

As with *esp*, *gelE* gene participation in biofilm formation is also controversial. Some authors have observed that, in fact, the presence of gelatinase enzyme can affect the virulence and the process of biofilm formation in *Enterococcus* (Dworniczek et al. 2012). However, other authors (Ballering et al. 2009) have demonstrated that *gelE* is not associated with such phenotypes. Nevertheless, our study demonstrated that gelatinase expression may have influenced biofilm formation.

AS is one of several *Enterococcus* adhesins that can be targeted for therapy against infectious endocarditis. The expression of *asa1* has an important effect on biofilm formation because this genotype promotes the accumulation of adherent microorganisms to a surface (Schlievert et al. 2010). One study identified several functional subdomains in the *Enterococcus asa1* gene and mutations in these *loci* drastically reduced the ability to form biofilm (Chuang-Smith et al. 2010). Our study corroborates these findings, since *asa1* was significantly more frequent in biofilm-producing strains.

However, based on our statistical analysis, biofilm formation could not be linked to any specific gene. In fact, this phenotype is multifactorial and depends on a number of genes working together along with extrinsic factors. So far, several other genes or gene sets have been reported as auxiliaries in biofilm formation in *Enterococcus*, which highlights the complexity and the multifactorial nature of this trait (Dworniczek et al. 2012).

Vancomycin resistance was not associated with more virulent strains in our study. Indeed, according to Giridhara et al. (2010), there is no significant difference in virulence factors, ability to cause infection or vancomycin susceptibility among *Enterococcus* isolates. Acquired resistance and virulence factors may compromise microorganism fitness. Thus, multiresistance characteristics in strains presenting restricted virulence arsenals are a common feature and are frequently seen in opportunistic pathogens (Foucault et al. 2010, Rigottier-Gois et al. 2011).

Molecular epidemiological studies have reported the spread, around the world, of a hospital-adapted complex of *E. faecium* designated epidemic clonal complex-17 (CC17), which is associated with the majority of hospital outbreaks and clinical infections on all continents. The global success of CC17 seems to have been facilitated by the cumulative acquisition of antibiotic resistance, putative virulence traits and the ability to acquire different mobile genetic elements (Deshpande et al. 2007). Although the *esp* gene seems to be a common characteristic of CC17 strains, its occurrence is not homogeneous (Brilliantova et al. 2010). Brilliantova et al. (2010) evaluated 129 *vanA*-positive *E. faecium* isolates, which included two major clones belonging to CC17. Virtually all isolates (91%) were positive for the *gelE* gene, in contrast to the 5% positive rate determined in our study. On the other hand, Brilliantova et al. (2010) did not observe any *asa1*-positive isolates, which is in accordance with our findings.

Our VRE_{fm} group presented some characteristics of CC17, such as resistance to ampicillin and non-susceptibility to quinolones (data not shown). Moreover, the

esp gene was highly prevalent, but not always present. Further studies must be done to determine the sequence type of our isolates.

VRE_f enterococci have been observed by virtually all authors to have a high prevalence of virulence determinants (Udo & Al-Sweih 2011, Hasani et al. 2012). Nevertheless, Sharifi et al. (2012) studied the distribution of virulence factors and they observed a high frequency of all virulence factors (including *gelE* and *asa1*) except *esp*, which reinforces the complexity of the virulence process in this genus.

Although one could expect that virulence traits change depending on the source of isolation, we did not find any such association. Dahlén et al. (2012) compared *E. faecalis* recovered from oral mucosal infections and deep infections and they did not find any discrepancies in the distribution of *asa1*, *gelE* or *esp* between both groups. Significant differences in the distribution of virulence factors are potentially achievable by comparing infecting and colonising isolates. However, Johansson and Rasmussen (2012) compared virulence factors (*ace*, *asa1*, *gelE*, *esp*) of *E. faecalis* from infective endocarditis and normal flora and observed that only biofilm formation had a significantly different occurrence (more pronounced in the normal flora group). Indeed, the source of isolation (including surveillance cultures) did not influence the virulence factor distribution among our enterococcal population.

In terms of molecular epidemiology, it was impossible to associate any virulence factor with any specific subclone of VRE_f or VRE_{fm}. On the other hand, among the highly heterogeneous population of VSE_f, the distribution of virulence factors was homogeneous, with most isolates presenting almost all the evaluated virulence determinants. Thus, molecular relatedness does not affect virulence gene distribution (Willems et al. 2005).

Our study has some limitations. The small number of isolates could have compromised the statistical analysis, as could the clonal relationship among them. Moreover, our isolates of VRE_{fm} and VRE_f were closely related and potentially do not represent the real distribution of the putative virulence factors in the enterococcal population. Studies with a higher number of heterogeneous VREs should be performed to clarify each one's role in enterococcal pathogenesis.

In the results presented here, *E. faecalis* and *E. faecium* isolates showed significantly different patterns of virulence determinants, which reinforces the findings of other authors (Hasani et al. 2012, Sharifi et al. 2012). In addition, neither the source of isolation nor the clonal relationship or vancomycin resistance influenced the distribution of virulence determinants.

Given the increasing importance of *Enterococcus* as nosocomial pathogens, the identification of virulence factors associated with invasiveness and disease severity has become an important subject of concern. The development of other mechanisms to control infection, such as preventing *Enterococcus* biofilm formation or inhibiting the action of other virulence factors, may provide an alternative method of therapy, especially considering that antimicrobial treatment is challenging for this genus.

REFERENCES

Ballering KS, Kristich CJ, Grindle SM, Oromendia A, Beattie DT 2009. Functional genomics of *Enterococcus faecalis*: multiple novel genetic determinants for biofilm formation in the core genome. *J Bacteriol 191*: 2806-2814.

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

Chuang ON, Schlievert PM, Wells CL, Manias DA, Tripp TJ 2009. Multiple functional domains of *Enterococcus faecalis* aggregation substance Asc10 contribute to endocarditis virulence. *Infect Immun 77*: 539-548.

Chuang-Smith ON, Wells CL, Henry-Stanley MJ, Dunny GM 2010. Acceleration of *Enterococcus faecalis* biofilm formation by aggregation substance expression in an ex vivo model of cardiac valve colonization. *PLoS ONE 5*: e15798.

CLSI - Clinical Laboratory Standards Institute 2011. *Performance standard for antimicrobial susceptibility testing*, Document M100-S21, CLSI, Wayne, 172 pp.

Dahlén G, Blomqvist S, Almstahl A, Carlén A 2012. Virulence factors and antibiotic susceptibility in enterococci isolated from oral mucosal and deep infections. *J Oral Microbiol 4*: 10855.

Deshpande LM, Fritsche TR, Moet GJ, Biedenbach DJ, Jones RN 2007. Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY Antimicrobial Surveillance Program. *Diagn Microbiol Infect Dis 58*: 163-170.

Dworniczek E, Piwowarczyk J, Bania J, Kowalska-Krochmal B, Walecka E, Seniuk A, Dolna I, Gosciniak G 2012. *Enterococcus* in wound infections: virulence and antimicrobial resistance. *Acta Microbiol Immunol Hung 59*: 263-269.

Eaton TJ, Gasson MJ 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl Environ Microbiol 67*: 1628-1635.

Fisher K, Phillips C 2009. The ecology, epidemiology and virulence of *Enterococcus*. *Microbiol 155*: 1749-1757.

Foucault ML, Depardieu F, Courvalin P, Grillot-Courvalin C 2010. Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci. *Proc Natl Acad Sci 107*: 16964-16969.

Garth AJ, Swogger E, Wolcott R, deLancey Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart OS 2008. Biofilms in chronic wounds. *Wound Repair Regen 16*: 37-44.

Giridhara Upadhyaya PM, Umapathy BL, Ravikumar KL 2010. Comparative study for the presence of enterococcal virulence factors gelatinase, hemolysin and biofilm among clinical and commensal isolates of *Enterococcus faecalis*. *J Lab Physicians 2*: 100-104.

Hasani A, Sharifi Y, Ghotaslou R, Naghili B, Hasani A, Aghazadeh M, Milani M, Mazmani A 2012. Molecular screening of virulence genes in high-level gentamicin-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from clinical specimens in Northwest Iran. *Indian J Med Microbiol 30*: 175-181.

Heikens E, Bonten MJ, Willems RJ 2007. Enterococcal surface protein Esp is important for biofilm formation of *Enterococcus faecium* E1162. *J Bacteriol 189*: 8233-8240.

Johansson D, Rasmussen M 2012. Virulence factors in isolates of *Enterococcus faecalis* from infective endocarditis and from the normal flora. *Microb Pathog 55*: 28-31.

Lindenstrau AG, Pavlovic M, Bringmann A, Behr J, Ehrmann MA, Vogel RF 2011. Comparison of genotypic and phenotypic cluster

analyses of virulence determinants and possible role of CRISPR elements towards their incidence in *Enterococcus faecalis* and *Enterococcus faecium*. *Syst Appl Microbiol 34*: 553-560.

Marra A, Dib-Hajj F, Lamb L, Kaczmarek F, Shang W, Beckius G, Millici AJ, Medina I, Gootz TD 2007. Enterococcal virulence determinants may be involved in resistance to clinical therapy. *Diagn Microbiol Infect Dis 58*: 59-65.

Rigottier-Gois L, Alberti A, Houel A, Taly JF, Palcy P 2011. Large-scale screening of a targeted *Enterococcus faecalis* mutant library identifies envelope fitness factors. *PLoS ONE 6*: e29023.

Ruzon FI, Paula SB, Kanoshiki RL, Santos JP, Kerbauy G, Kobayashi RKT, Yamauchi LM, Perugini MRE, Yamada-Ogatta SF 2010. Virulence determinants in vancomycin-resistant *Enterococcus faecium vanA* isolated from different sources at University Hospital of Londrina, Paraná, Brazil. *J Microbiol 48*: 814-821.

Schlievert PM, Chuang-Smith ON, Peterson ML, Cook LC, Dunny GM 2010. *Enterococcus faecalis* endocarditis severity in rabbits is reduced by IgG Fabs interfering with aggregation substance. *PLoS One 5*: e13194.

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

Sillanpää J, Nallapareddy SR, Singh KV, Prakash VP, Fothergill T, Ton-That H, Murray BE 2010. Characterization of the ebpfm pilus-encoding operon of *Enterococcus faecium* and its role in biofilm formation and virulence in a murine model of urinary tract infection. *Virulence 1*: 236-246.

Stepanovic S, Vucovic D, Savic B, Svabic-Vlahovic M 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods 40*: 175-179.

Teixeira LM, Carvalho MGS, Merquior VLC, Steigerwalt AG, Brenner DJ, Facklam RR 1997. Phenotypic and genotypic characterization of *Vagococcus fluvialis*, including strains isolated from human sources. *J Clin Microbiol 35*: 2778-2781.

Teixeira LM, Carvalho MGS, Shewmaker PL, Facklam RR 2011. *Manual of clinical microbiology*, 10th ed., American Society for Microbiology, Washington DC, 2630 pp.

Tsikrikonis G, Maniatis AN, Labrou M, Ntokou E, Michail G, Daponte A, Stathopoulos C, Tsakris A, Pournaras S 2012. Differences in biofilm formation and virulence factors between clinical and fecal enterococcal isolates of human and animal origin. *Microb Pathog 52*: 336-343.

Udo EE, Al-Sweih N 2011. Frequency of virulence-associated genes in *Enterococccus faecalis* isolated in Kuwait hospitals. *Med Princ Pract 20*: 259-264.

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

Willems RJL, Bonten MJM 2007. Glycopeptide-resistant enterococci: deciphering virulence, resistance and epidemicity. *Curr Opin Infect Dis 20*: 384-390.