The Journal of Microbiology (2010) Vol. 48, No. 6, pp. 814-821
DOI 10.1007/s12275-010-0099-5

Virulence Determinants in Vancomycin-Resistant *Enterococcus faecium van*A Isolated from Different Sources at University Hospital of Londrina, Paraná, Brazil

Flávia Imanishi Ruzon[1], Suelen Balero de Paula[1], Renata Lumi Kanoshiki[1], Jussevania Pereira-Santos[1], Gilselena Kerbauy[1], Renata Katsuko Takayama Kobayashi[2], Lucy Megumi Yamauchi[1], Márcia Regina Eches Perugini[3], and Sueli Fumie Yamada-Ogatta[1]*

[1]Laboratório de Biologia Molecular de Microrganismos, [2]Laboratório de Bacteriologia, Departamento de Microbiologia, Centro de Ciências Biológicas, [3]Laboratório de Microbiologia Clínica, Departamento de Patologia, Análises Clínicas e Toxicológicas, Universidade Estadual de Londrina, Londrina, Paraná 86051-990, Brazil

(Received March 15, 2010 / Accepted July 29, 2010)

***Enterococcus faecium*, especially those showing multidrug resistance, has emerged as a significant cause of healthcare-associated infections worldwide. However, relatively little is known about the virulence and pathogenesis of this species. The aim of this study was to determine the occurrence of four putative virulence determinants of *E. faecium* and to correlate them with phenotypic traits. Using forty *E. faecium van*A-type isolates from hospitalized patients and their environmental vicinity, we determined the following: the antimicrobial susceptibility profile, occurrence of the genes *cyl*A, *efa*A, *esp*, and *gel*E, hemolytic and gelatinase activities, capacity to form biofilm and *in vitro* adhesion to epithelial cells. All isolates were shown to be resistant to vancomycin and teicoplanin, as well as to two or more other antimicrobials. All isolates harbored at least one putative virulence marker, and the prevalence was as follows: *esp*, 87.5%; *efa*A, 82.5%; *gel*E, 70%; and *cyl*A, 65%. The presence of 4 genes was observed in 32.5% isolates. The presence of the *efa*A was associated with the presence of *esp*, regardless of the source of the isolates. A positive association with the presence of *cyl*A and hemolytic activity in the sheep blood agar assay was observed. No association was found for *gel*E and gelatinase production in the agar plate assay, for *efa*A and LLC-MK2 cell adhesion, and for *esp* and biofilm formation on polystyrene surface. These results show the presence of putative virulence genes in multiple antimicrobial resistant *E. faecium* isolates from different sources in a hospital setting.**

***Keywords*:** *E. faecium*, antimicrobial resistance, *van*A genotype, virulence factors

Enterococci are opportunistic pathogens that can cause a wide variety of diseases, especially in immunocompromised patients and healthcare-associated settings, such as urinary tract infections, surgical and burn wound infections, bacteremia and endocarditis (Sood *et al*., 2008). *Enterococcus faecalis* has been regarded as the most common causative agent of enterococcal infections (Titze-de-Almeida *et al*., 2004; d'Azevedo *et al*., 2006). However, *Enterococcus faecium* has become a significant cause of healthcare-associated infections worldwide (Treitman *et al*., 2005; Deshpande *et al*., 2007; Hoshuyama *et al*., 2008; Top *et al*., 2008; Werner *et al*., 2008). This scenario is likely explained in part by the capacity of *E. faecium* to acquire multiple antimicrobial resistance determinants (Willems and Bonten, 2007; Sood *et al*., 2008). Indeed, data from healthcare-associated infection surveillance all over the world have shown a growing percentage of vancomycin-resistant *E. faecium* (EVRfm) isolates (Treitman *et al*., 2005; Biedenbach *et al*., 2007; Deshpande *et al*., 2007).

Various traits of enterococci have been considered putative factors of virulence and infection development, and several investigators have detected the presence of the genes encoding putative virulence markers in *E. faecium* from different sources (Vankerckhoven *et al*., 2004, 2008; Camargo *et al*., 2006; Biavasco *et al*., 2007; Billström *et al*., 2008; Worth *et al*., 2008; Hällgren *et al*., 2009). A 37-kDa cell wall protein encoded by the *efa*A gene (*E. faecalis* antigen A) was first detected in the serum of a patient with *E. faecalis* endocarditis. The amino acid sequence of EfaA was found to show homology with a group of streptococcal proteins with adhesion properties (Lowe *et al*., 1995). Studies with *E. faecalis efa*A$^-$ mutant in a mouse peritonitis model suggested that EfaA is a virulence factor (Singh *et al*., 1998a). The *esp* gene encoding an enterococcal surface protein is located on a pathogenicity island in both *E. faecalis* (Shankar *et al*., 2002) and *E. faecium* (Leavis *et al*., 2004). In *E. faecalis*, *esp* has been identified among human- and animal-derived isolates (Hammerum and Jensen, 2002; Bittencourt de Marques and Suzart, 2004), but in *E. faecium*, it is specifically enriched in hospital-acquired isolates (Willems *et al*., 2001; Hammerum and Jensen, 2002). The presence of the gene *esp* has been associated with colonization and persistence of *E. faecalis* in ascending urinary tract infection (Shankar *et al*., 2001). Some secreted molecules are putative virulence factors of enterococci. Gelatinase is an extracellular zinc metalloprotease encoded by the chromosomal *gel*E gene (Su *et al*., 1991), whose product can hydrolyze gelatin, casein, hemoglobin, fibrin, and small peptides (Mäkinen *et al*., 1989; Su *et al*., 1991; Waters *et al*., 2003). It also cleaves C3a and C3b components of the human complement system (Park

* For correspondence. E-mail: ogatta@uel.br; Tel: +55-43-3371-4297; Fax: +55-43-3371-4788

et al., 2007), which can explain the complement resistance of *E. faecalis*. In addition, gelatinase activity has been shown to contribute to virulence in a mouse peritonitis model (Singh *et al*., 1998b). Another secreted virulence determinant is cytolysin, which is a two-peptide lytic toxin encoded by an operon consisting of eight genes (*cyl*R1, *cyl*R2, *cyl*L_L, *cyl*L_S, *cyl*M, *cyl*B, *cyl*A, *cyl*I) carried on a plasmid or integrated into the bacterial chromosome (Shankar *et al*., 2002; Coburn and Gilmore, 2003). Two main activities are associated with cytolysin, hemolysin, and bacteriocin: they can lyse erythrocytes of different origin (Izumi *et al*., 2005) and a broad range of Gram-positive bacteria (Coburn and Gilmore, 2003). Cytolysin contributes to enterococcal virulence as shown in several animal models of enterococcal infections (Jett *et al*., 1992; Singh *et al*., 1998b).

In this study, the occurrence of four virulence determinants in VREfm isolates from hospitalized patients and their environmental vicinity was investigated by molecular and phenotypic methods.

Materials and Methods

Microorganisms

At University Hospital of Londrina, Paraná, Brazil, from 2002 to 2007, VREfm was the most frequent species isolated from infected and colonized patients and environmental sources (Dr. Perugini MRE, personal communication). A total of 40 non-duplicate VREfm isolates of different origin were randomly taken from the bacterial collection of the Laboratory of Clinical Microbiology of Universidade Estadual de Londrina, Londrina, PR, Brazil. Human isolates were classified according to CDC definitions of healthcare-associated infections (Horan *et al*., 2008). A total of 30 isolates were recovered from different patients: 18 isolates were considered colonizing enterococci, where 10 were isolated from rectal swab specimens (fecal carriage) and 8 from urine. Twelve human isolates were recovered from various clinical sources (mainly urine). Ten isolates were obtained by rubbing pre-moistened swabs over the sites in the immediate vicinity of the patient and the general areas in patients' rooms. All enterococci were identified to the species level on the basis of the profile generated by the automated MicroScan WalkAway 96 Instrument (Dade MicroScan, USA). Concomitantly, colony morphology, Gram stain, catalase assay, tolerance to bile-esculin, growth in 6.5% NaCl were also determined and biochemical tests performed using the API 20S (bioMérieux, Brazil). Bacteria were kept in brain heart infusion (BHI, Himedia, India) agar medium at room temperature and also preserved in 20% glycerol-BHI broth at -20°C.

DNA extraction

Whole DNA of all enterococcal strains was extracted by the boiling method as described in Bittencourt de Marques and Suzart (2004). Briefly, a single bacterial colony was added to 3 ml BHI broth and incubated at 37°C for 18 h. The cultures were centrifuged at 10,000×g for 5 min, the bacterial pellets were resuspended in 300 µl sterile ultrapure water and boiled (100°C) for 30 min. Cellular debris was removed by centrifugation and a 10-µl aliquot of supernatant was used in all amplification reactions.

Antimicrobial susceptibility and vancomycin resistance genes

The isolates were tested for antimicrobial susceptibility to 9 agents (ampicillin, ciprofloxacin, erythromycin, gentamicin, rifampicin, streptomycin, teicoplanin, tetracycline, and vancomycin), using the automated broth microdilution panel of the MicroScan WalkAway 96 Instrument, according to the manufacturer's recommendations. The results reported here were those recorded after 24 h of incubation. The susceptibility breakpoints used were those recommended by the Clinical Laboratory Standard Institute (2007). *E. faecalis* ATCC 29212 and 51299 were used for quality control. The vancomycin resistance gene was determined using multiplex PCR as described by Petrich *et al*. (1999). The primer nucleotide sequences are shown in Table 1.

PCR primer design and amplification of putative virulence genes

The genes encoding putative virulence factors tested were as follows: *cyl*A (activator of cytolysin, a secreted protein with hemolysin/bacteriocin activities), *efa*A (*E. faecalis* antigen A, an endocarditis-associated virulence factor), *esp* (enterococcal surface protein), and

Table 1. Description of primers used in PCR for the detection of putative virulence markers and vancomycin-resistance genes of *Enterococcus faecium* from different sources

Target gene[a]	Sequence of the primer (5′→3′)	Amplicon size (bp)	Accession number[b]
*cyl*A	F: TAAGGTGATGGATGGGACAGATG	216	L37110.1
	R: GCGACTCATTTCCTGCTGATG		
*efa*A	F: TCGTACCAGTCGGAACAGATCCGCAT	235	AF042288
	R: GGTGAAGAACATACAAAAGCGGATCC		
esp	F: CTAATGCAAGTCCACGTCCAGTCG	243	AY322499
	R: GTGATGGAAACCCTGACGATAAAGAAG		
*gel*E	F: CGCCAAGTAAACACGGACAACCAGA	247	M37185
	R: GTGATGCGATGCTTGCTGCTGC		
*van*A	F: GCTGCGATATTCAAAGCTCA	545	*
	R: CAGTACAATGCGGCCGTTA		
*van*B	F: ATGGGAAGCCGATAGTCTC	368	*
	R: GTTACGCCAAAGGACGAAC		

[a] *cyl*A, activator of cytolysin; *efa*A, *E. faecalis* antigen A; *esp*, enterococcal surface protein; *gel*E, gelatinase; *van*A and *van*B, vancomycin resistance type A and B, respectively.
[b] The nucleotide sequences of *E. faecium* genes deposited in the GenBank/EMBL databases used for specific primer design. * According to Petrich *et al*. (1999).

*gel*E (gelatinase). The nucleotide sequences of *E. faecium* genes deposited in the GenBank/EMBL databases were used for specific primer design with Primer Select software (DNASTAR Lasergene). The primer sequences and expected size of amplicons for each PCR assay are shown in Table 1. PCR was performed in a final volume of 20 µl containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each forward and reverse primer, 2.5 U *Taq* DNA polymerase (Invitrogen, Brazil), and 10 µl of genomic DNA. The amplification reactions were performed in a MWG Biotech Primus Thermal Cycler with an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec and an extension step at 72°C for 1 min. Negative control reactions without any template DNA were carried out simultaneously. After amplification, 5 µl of each PCR sample and a 100-bp DNA molecular weight ladder were separated in a 2.0% agarose gel (Invitrogen) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 4.8 V/cm for 2 h, and the gel was stained with ethidium bromide. Positive controls in the PCR assays were *E. faecalis* 206 (*efa*A), *E. faecalis* 316 (*esp*, *cyl*A), and *E. faecalis* 357 (*gel*E) (Bittencourt de Marques and Suzart, 2004).

Detection of gelatinase and hemolytic activities

Gelatinase and hemolytic activities were assayed on agar plates containing 1% gelatin (Difco, USA) and 5% fresh sheep blood as substrate, respectively. For both assays, enterococcal strains were previously cultured at 37°C for 18 h in BHI broth. For gelatinase activity, a 10-µl suspension of 10^7 cells was placed on the surface of nutrient agar medium supplemented with 1% gelatin, and the cultures were incubated at 37°C for 24 h. Afterward, the presence of the degradation zone around the colony was determined. For hemolytic activity, bacterial cells were added to the surface of Muller Hinton agar (Himedia, India) medium supplemented with 5% fresh sheep blood, pH 7.3. The plates were incubated at 37°C in 5% CO_2 for 48 h. Hemolytic activity was indicated by a translucent halo around the inoculum site. Each isolate was tested in triplicate, and the experiments were carried out on three different occasions. *Serratia marcescens* and *Staphylococcus aureus* ATCC 25923 were used as positive controls for gelatinase and hemolysin activities, respectively.

Biofilm formation on polystyrene surface

Enterococcal strains were previously grown at 37°C for 24 h in BHI broth supplemented with 1% glucose. The cell density was adjusted to 1.5×10^8 CFU/ml in the same medium, and a 200-µl aliquot of each suspension was transferred to two wells of a 96-well flat-bottomed polystyrene microtiter plate (Techno Plastic Products, Switzerland). Negative control wells containing only broth were included. The plates were incubated statically under aerobic conditions at 37°C for 24 h. After the incubation period, the medium was aspirated off and non-adherent cells were removed by washing thoroughly three times with sterile distilled water. The adherent bacterial film was fixed by air drying at 60°C for 1 h and then stained with 200 µl of filtered 2% crystal violet (Gram stain). The optical density of each well was measured at 570 nm using a microtiter plate reader (Universal Microplate Reader ELx 800, Bio-Tek Instruments). Experiments were carried out in triplicate on three different occasions. Biofilm formation was scored according to the criteria proposed by Stepanovic *et al*. (2000).

Adhesion of bacterial strains to LLC-MK2 cells

Enterococcal strains were tested for adherence to LLC-MK2 (rhesus monkey kidney) cells as described by Archimbaud *et al*. (2002), with minor modifications. LLC-MK2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B in a humidified, 5% CO_2 atmosphere at 37°C. For adhesion assays, LLC-MK2 cells were seeded in 24-well plates at 1×10^5 cells per well and incubated for 18 h. The medium was removed and replaced with fresh culture medium minus the antimicrobials, the wells were inoculated with *E. faecium* strains with approximately 1×10^7 cells, and the plates were incubated at 37°C for 2 h in a 5% CO_2 atmosphere. Non-adherent bacterial cells were removed by washing with sterile phosphate-buffered saline (PBS). Adherent bacteria were harvested by treatment of the cell monolayers with 1 ml 0.5% (v/v) Triton X-100 (Sigma Chemical Co.) for 10 min on ice. The viable bacteria were enumerated by dilution plating in BHI agar. Experiments were carried out in duplicate on three different occasions. The percent adherence was calculated by the equation: % Adherence = $(CFU_{120}/CFU_0)\times100$, where CFU_{120} refers to adhered bacterial cells per ml after 2 h and CFU_0 the initial number of inoculated cells. A percent adherence 1.5 times higher than the mean % adherence of all isolates was considered significant.

Statistical analyses

The presence of the virulence markers regarding the origin of the *E. faecium* isolates was analyzed by the Fisher method. Spearman's rank correlation was determined to compare the degree of association between the presence of virulence genes and the corresponding phenotype. A p value less than 0.05 was considered significant.

Results

Phenotypic and genotypic characterization of antimicrobial susceptibilities

Using the automated broth microdilution system, an antimicrobial susceptibility profile for 9 agents was determined. All isolates were resistant to vancomycin and teicoplanin, and the mechanism of resistance was mediated by the *van*A type gene. Besides being resistant to vancomycin and teicoplanin, all isolates showed resistance to two or more other antimicrobials (Table 2). According to the phenotypic resistance profile, the *E. faecium* isolates were classified into 8 groups (Table 2). The majority of the isolates (22 out of 40 isolates, 55%) displayed the group II phenotypic antimicrobial resistance profile (ampicillin, ciprofloxacin, erythromycin, tetracycline, teicoplanin, and vancomycin).

Detection of putative virulence genes

The presence of the genes *cyl*A, *efa*A, *esp*, and *gel*E in *E. faecium* isolates was determined by PCR, and the results are shown in Table 3. All virulence genes examined were commonly found in *E. faecium* isolates from different sources, and the prevalence was as follows: *esp*, 87.5%; *efa*A, 82.5%; *gel*E, 70%; and *cyl*A, 65%. All isolates harbored at least one putative virulence marker and the presence of 4 genes was observed in 32.5% isolates (Table 4). Regarding the origin of the isolates, only the presence of the *gel*E gene was signifycantly higher in colonization and infection isolates compared to environmental isolates ($p<0.01$). In addition, the presence of the *efa*A gene was associated with the presence of the *esp* gene, regardless of the source of the isolates ($p<0.001$).

Table 2. Phenotypic antimicrobial resistance profile of *Enterococcus faecium* isolated from different sources at University Hospital of Londrina

Group	Phenotypic antimicrobial resistance	Source[a]		
		Environment	Colonization	Infection
I	amp, cip, eri, gen, rif, tei, van	6 (60)	6 (33.4)	-
II	amp, cip, eri, tei ,tet, van	2 (20)	11 (61.1)	9 (75)
III	amp, cip, eri, str, tei, tet, van	-	-	1 (8.3)
IV	amp, eri, gen, rif, tei, van	1 (10)	-	-
V	amp, cip, eri, gen, rif, tei, tet, van	-	-	1 (8.3)
VI	rif, tei, van	-	1 (5.6)	-
VII	amp, cip, eri, tei, van	-	-	1 (8.3)
VIII	eri, gen, rif, tei, tet, van	1 (10)	-	-
Total		10	18	12

[a] Represents the number of isolates showing the profile indicated (percentage of the total in each source category). amp, ampicillin; cip, ciprofloxacin; eri, erythromycin; gen, gentamicin; rif, rifampicin; str, streptomycin; tei, teicoplanin; tet, tetracycline; van, vancomycin; -, none.

Relationship between virulence genes and putative corresponding phenotypes

A positive association with the presence of *cyl*A gene and hemolytic activity in the sheep blood agar assay was observed ($p<0.001$). Seventeen enterococcal isolates harbored the gene *cyl*A and formed a translucent halo around the inoculum site characteristic of beta-hemolysis, and 13 *cyl*A-negative isolates did not show hemolysis on sheep-blood agar medium. Nine isolates were positive for the presence of the gene but were negative for hemolytic activity. One isolate did not possess the gene and was positive for hemolytic activity. No significant association was found for *gel*E gene and gelatinase production in the agar plate assay. In 23 isolates, the presence of the gene was associated with gelatin degradation. Fifteen isolates were positive for the presence of *gel*E gene but were unable to use gelatin as a substrate. Two isolates degraded gelatin, but did not possess the *gel*E gene. Also, no significant association was found between the presence of *efa*A gene and adhesion to LLC-MK2 cells. Nine isolates harboring *efa*A were able to adhere to these cells, and in 3 adherent isolates the gene was not detected in their genome. A similar result was obtained for the presence of the *esp* gene and biofilm formation. Only 11 isolates harboring the *esp* were able to form biofilm on the surface of a polystyrene microplate. A total of 28 isolates possessed the *esp* gene and did not form biofilm, and in contrast, the gene was not detected in the genome of 1 isolate that was able to form biofilm on that surface. A significant correlation was observed between cell adhesion and biofilm formation ($p<0.02$). Table 5 summarizes all results obtained in this study.

Table 3. Frequency of putative virulence genes distributed according to source of the *Enterococcus faecium* isolates

Virulence marker[a]	Source[b]			*E. faecium* isolates[c]
	Environment $n=10$	Colonization $n=18$	Infection $n=12$	
*cyl*A	7 (70)	13 (72.2)	6 (50)	26 (65)
*efa*A	9 (90)	14 (77.8)	10 (83.3)	33 (82.5)
esp	10 (100)	14 (77.8)	11 (91.7)	35 (87.5)
*gel*E	3* (30)	15 (83.3)	10 (83.3)	28 (70)

[a] *cyl*A, activator of cytolysin; *efa*A, *E. faecalis* antigen A; *esp*, enterococcal surface protein; *gel*E, gelatinase.
[b] Represents the number of isolates harboring the gene (percentage of the total in each source category).
[c] Represents the number of isolates harboring the gene regardless of the source (percentage of the total isolates).
* Significantly different ($p<0.01$) when compared to colonization and infection isolates.

Discussion

Over the past two decades, studies on the putative virulence factors of enterococci have mainly focused on *E. faecalis* isolates, the most frequent agent of enterococcal infections. However, as mentioned, the ratio of *E. faecalis* to *E. faecium* human infections has been changing all over the world and *E. faecium* isolates, especially those showing multidrug resistance, are responsible for the leading causes of healthcare-associated infections (Treitman *et al*., 2005; Biedenbach *et al*., 2007; Deshpande *et al*., 2007; Hoshuyama *et al*., 2008; Top *et al*., 2008). VREfm in hospital wards, due to the presence of fecal carriage or infected patients, and contaminated environmental surfaces and medical equipment, has been considered a potential risk factor for enterococcal transmission (Drees *et al*.,

Table 4. *Enterococcus faecium* isolates harboring clusters of virulence genes according to their origin

Virulence markers[a]	Source[b]			Total of *E. faecium* isolates (%)
	Environment $n=10$	Colonization $n=18$	Infection $n=12$	
*cyl*A, *efa*A, *esp*, *gel*E	1 (10)	9 (50)	3 (25)	13 (32.5)
*cyl*A, *esp*, *gel*E	1 (10)	-	2 (16.7)	3 (7.5)
*cyl*A, *efa*A, *esp*	4 (40)	1 (5.6)	1 (8.3)	6 (15)
*cyl*A, *gel*E	-	3 (16.7)	1 (8.3)	4 (10)
*efa*A, *esp*, *gel*E	1 (10)	2 (11.1)	4 (33.3)	7 (17.5)
*efa*A, *esp*	3 (30)	2 (11.1)	1 (8.3)	6 (15)
*gel*E	-	1 (5.6)	-	1 (2.5)

[a] *cyl*A, activator of cytolysin; *efa*A, *E. faecalis* antigen A; *esp*, enterococcal surface protein; *gel*E, gelatinase.
[b] Represents the number of isolates harboring the gene clusters (percentage of the total in each source category).

Table 5. Characteristics of the *Enterococcus faecium* isolates recovered from different sources at University Hospital of Londrina, Paraná

Isolate[a]	Resistance[b]	Virulence markers[c]							
		*cyl*A	Hemolysin	*efa*A	Adhesion	*esp*	Biofilm	*gel*E	Gelatinase
E									
150	I	+	-	+	+	+	-	-	-
151	I	+	-	+	-	+	+	-	+
152	I	-	-	+	-	+	+	-	-
153	I	+	-	+	-	+	-	-	-
154	II	+	+	+	-	+	-	-	-
155	IV	-	-	+	-	+	-	-	-
156	VIII	+	-	+	-	+	-	+	-
157	I	+	-	-	+	+	-	+	-
173	I	+	-	+	-	+	-	-	-
174	II	-	-	+	-	+	-	+	-
C									
15	II	+	+	+	-	+	-	+	-
22	II	-	-	+	+	+	-	-	-
31	II	+	-	+	+	+	-	+	-
36	II	-	-	-	-	-	-	+	+
43	II	+	+	+	-	+	-	+	+
44	II	+	+	+	-	+	-	+	+
48	II	-	-	+	-	+	-	+	+
158	I	-	-	+	+	+	+	+	+
159	I	+	+	+	+	+	+	+	+
160	I	+	+	+	+	+	+	+	-
161	I	+	-	+	+	+	+	+	-
162	II	+	+	-	+	-	+	+	-
163	I	+	+	+	-	+	-	+	-
164	II	+	+	-	-	-	-	+	-
166	I	+	-	-	-	-	-	+	+
168	VI	-	-	+	+	+	-	-	-
175	II	+	+	+	+	+	-	-	-
176	II	+	+	+	-	+	-	-	-
I									
5	II	+	+	+	-	+	-	+	+
17	VII	+	+	-	-	+	-	+	+
28	II	-	+	+	-	+	-	+	-
35	II	+	+	+	-	+	-	-	-
37	II	+	+	+	-	+	-	+	-
47	II	-	-	+	-	+	-	-	+
80	II	+	+	+	-	+	-	+	-
167	II	-	-	+	-	+	-	+	-
169	II	-	-	+	-	+	-	+	-
170	III	-	-	+	-	+	+	+	+
171	V	+	+	-	+	-	-	+	-
172	II	-	-	+	-	+	-	+	+

[a] Source of isolate: E, environment; C, colonization; I, infection.
[b] Group of antimicrobial resistance profile.
[c] Symbols represent: +, presence/expression of virulence marker; -, absence of virulence marker.

2008).

In this study, the presence of four virulence markers and the corresponding putative phenotype was determined in *E. faecium* isolates from colonized and infected patients and from environmental surfaces in the patient's room. A high frequency of multidrug resistant *E. faecium* isolates was observed, and this phenotype was independent of the origin. Resistance to vancomycin was common to all isolates, and they harbored the *van*A gene. This result is in accordance with the glycopeptide-resistance phenotype detected by the broth microdilution assay, where all isolates were resistant to high levels of vancomycin and teicoplanin. The mechanisms of vancomycin resistance mediated by the *van*A and *van*B genes are more widely distributed (Sood *et al*., 2008; Werner *et al*., 2008; Dendle *et al*., 2009; Chang *et al*., 2010). However, discrepant results have been obtained with regard to the vancomycin-resistance phenotype and genotype in enterococcal isolates. Henrique *et al*. (2008) described that *E. faecalis* and *E. faecium* isolates harbored the *van*A gene and showed vancomycin resistance but were susceptible or moderately resistant to teicoplanin. In addition, isolates harboring both the *van*A and *van*B genes with the VanA phenotype were also detected (Dendle *et al*., 2009).

Although *E. faecium* is considered an important healthcare-associated pathogen, little is known about its virulence. In this study, at least one virulence marker analyzed was detected by PCR in all *E. faecium* isolates. Several screenings using *E. faecium* isolates from different sources have shown the low prevalence of the virulence markers in this species. Overall, only the *esp* gene was frequently found in these studies followed by the *hyl* gene which encodes hyaluronidase (Vankerckhoven *et al*., 2004, 2008; Camargo *et al*., 2006; Billström *et al*., 2008; Worth *et al*., 2008; Hällgren *et al*., 2009; Chang *et al*., 2010).

The *esp* gene was detected in the majority of the VREfm isolates in this study, consistent with the findings of others (Camargo *et al*., 2006; Chang *et al*., 2010). However, there was no difference regarding the origin of the enterococci, as shown by Vankerckhoven *et al*. (2004). In that study, the presence of the gene *esp* was significantly higher in VREfm (predominantly *van*A type) isolated from clinical specimens compared to fecal carriage patients. There was no correlation with the presence of *esp* gene and biofilm formation on polystyrene surface in our isolates, where the rate was low in all cases. As demonstrated, Esp expression on the surface of *E. faecium* is variable among strains and depends on growth conditions, with elevated expression at 37°C compared to 21°C, and when grown under anaerobic compared to aerobic conditions. In addition, Esp expression is correlated with initial adherence and biofilm formation on polystyrene surface (Van Wamel *et al*., 2007). In view of this, other surfaces and conditions should be assayed to evaluate the capacity of these isolates to form biofilm.

On the other hand, there is controversy over the role of *E. faecalis* Esp in biofilm formation. While some investigators found no correlation between the presence or absence of the *esp* gene in clinical isolates and biofilm formation (Kristich *et al*., 2004), Esp-mediated biofilm formation was demonstrated by others (Toledo-Arana *et al*., 2001; Tendolkar *et al*., 2004). Esp expression on the surface of *E. faecium* seems to play a role in mediating initial adherence and biofilm formation on polystyrene surface (Van Wamel *et al*., 2007). This was corroborated by Heikens *et al*. (2007), who demonstrated that adherence and biofilm formation, on the same surface, were significantly reduced in an *E. faecium esp*$^-$ mutant compared to wild-type bacteria. In contrast, there were no significant differences between *E. faecium* esp$^-$ and the parental strain in adherence to Caco-2 cells and intestinal colonization of mice. These results indicate that Esp expression is not essential for these processes (Heikens *et al*., 2009).

A positive correlation was observed for the presence of the *esp* gene and *efa*A gene, the second most frequent gene found in our isolates. This is in contrast to the results of Billtröm *et al*. (2008), who did not detect the presence of the *efa*A gene in *E. faecium* isolates from the blood of hospitalized patients.

Most of the isolates harbored the *gel*E gene in this study. This is in contrast to the findings of Camargo *et al*. (2006). These authors did not detect *gel*E in any isolates of VREfm and vancomycin-sensitive *E. faecium* from different sources. No gelatinase activity was detected in 15 *E. faecium gel*E-positive isolates. Similar results were obtained by Biavasco *et al*. (2007) in VREfm *van*A type isolates from human and animal feces. As gelatinase can degrade different peptides besides gelatin (Mäkinen *et al*., 1989; Su *et al*., 1991; Waters *et al*., 2003), other substrates should be evaluated for gelatinase production.

The gene encoding the cytolysin activator *cyl*A was found in 50% of the isolates in our study. This is in disagreement with those results reported by several authors (Vankerckhoven *et al*., 2004, 2008; Camargo *et al*., 2006; Billström *et al*., 2008; Worth *et al*., 2008; Hällgren *et al*., 2009), who did not find any gene from the *cyl* operon in *E. faecium* isolates. We found a significant correlation between the presence/absence of *cyl*A gene and positive/negative hemolysin activity in the sheep blood agar assay, corroborating the results of Biavasco *et al*. (2007). These investigators did not detect the *cyl*B gene in hemolysin-negative *E. faecium* isolates. The gene product in 9 *cyl*A-positive isolates was probably related to bacteriocin activity.

One important aspect of enterococci is that several virulence markers and genes encoding antimicrobial resistance are located on mobile elements (Shankar *et al*., 2002; Leavis *et al*., 2004) and could be transferred among themselves and to other bacteria (Coburn *et al*., 2007; Werner *et al*., 2008). The surveillance strategies to limit the spread of multidrug-resistant *E. faecium* and other enterococci are needed in any hospital. We agree with Top *et al*. (2008) that knowledge about the virulence of these microorganisms can contribute to the development of new strategies in fighting enterococcal infection.

In conclusion, we found a high prevalence of *E. faecium* isolates harboring multiple antimicrobial resistance and putative virulence genes, among isolates that were recovered from different sources at University Hospital of Londrina. Besides being resistant to vancomycin and teicoplanin, all isolates showed resistance to two or more other antimicrobials. At least one out of four genes (*cyl*A, *efa*A, *esp*, and *gel*E) was commonly found in *E. faecium* isolates, but overall, there were no correlations with the putative phenotype assayed, except for *cyl*A gene and hemolytic activity. Further studies are

warranted to evaluate the role of these virulence markers in the pathogenesis of *E. faecium*, and such investigations are currently underway in our laboratory.

Acknowledgements

This work was supported by grants from Pro-Reitoria de Pesquisa e Pós-Graduação (PROPPG) of Universidade Estadual de Londrina (UEL). This work was part of the M.Sc. dissertation of F.I. Ruzon. We thank Dr. A. Leyva for English editing of the manuscript and Ediel Clementino da Costa for technical support.

References

Archimbaud, C., N. Shankar, C. Forestier, A. Baghdayan, M.S. Gilmore, F. Charbonné, and B. Joly. 2002. *In vitro* adhesive properties and virulence factors of *Enterococcus faecalis* strains. *Res. Microbiol*. 153, 75-80.

Biavasco, F., G. Foglia, C. Paoletti, G. Zandri, G. Magi, E. Guaglianone, A. Sundsfjord, C. Pruzzo, G. Donelli, and B. Facinelli. 2007. VanA-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl. Environ. Microbiol.* 73, 3307-3319.

Biedenbach, D.J., J.M. Bell, H.S. Sader, T.R. Fritsche, R.N. Jones, and J.D. Turnidge. 2007. Antimicrobial susceptibility of Gram-positive bacterial isolates from the Asia-Pacific region and an *in vitro* evaluation of the bactericidal activity of daptomycin, vancomycin, and teicoplanin: a SENTRY Program Report (2003-2004). *Int. J. Antimicrob. Agents* 30, 143-149.

Billström, H., B. Lund, A. Sullivan, and C.E. Nord. 2008. Virulence and antimicrobial resistance in clinical *Enterococcus faecium*. *Int. J. Antimicrob. Agents* 32, 374-377.

Bittencourt de Marques, E. and S. Suzart. 2004. Occurrence of virulence-associated genes in clinical *Enterococcus faecalis* strains isolated in Londrina, Brazil. *J. Med. Microbiol*. 53, 1069-1073.

Camargo, I.L., M.S. Gilmore, and A.L. Darini. 2006. Multilocus sequence-typing and analysis of putative virulence factors in vancomycin-resistant and vancomycin-sensitive *Enterococcus faecium* isolates from Brazil. *Clin. Microbiol. Infect*. 12, 1123-1130.

Chang, C.M., L.R. Wang, H.C. Lee, N.Y. Lee, C.J. Wu, and W.C. Ko. 2010. Characterization of vancomycin-resistant enterococci from hospitalized patients at a tertiary centre over a seven-year period. *J. Hosp. Infect.* doi: 10.1016/j.jhin.2009.10.025.

CLSI - Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 2007. Approved standard M7-A7. Wayne, PA, USA.

Coburn, P.S., A.S. Baghdayan, G.T. Dolan, and N. Shankar. 2007. Horizontal transfer of virulence genes encoded on the *Enterococcus faecalis* pathogenicity island. *Mol. Microbiol*. 63, 530-544.

Coburn, P.S. and M.S. Gilmore. 2003. The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cell Microbiol.* 5, 661-669.

d'Azevedo, P.A., C.A. Dias, and L.M. Teixeira. 2006. Genetic diversity and antimicrobial resistance of enterococcal isolates from Southern region of Brazil. *Rev. Inst. Med. Trop. São Paulo* 48, 11-16.

Dendle, C., S.A. Ballard, E.A. Grabsch, W. Gao, and M.L. Grayson. 2009. Outbreak of vancomycin-resistant *Enterococcus faecium* containing both *van*A and *van*B gene clusters. *J. Hosp. Infect*. 71, 379-381.

Deshpande, L.M., T.R. Fritsche, G.J. Moet, D.J. Biedenbach, and R.N. Jones. 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.

Drees, M., D.R. Snydman, C.H. Schmid, L. Barefoot, K. Hansjosten, P.M. Vue, M. Cronin, S.A. Nasraway, and Y. Golan. 2008. Prior environmental contamination increases the risk of acquisition of vancomycin-resistant enterococci. *Clin. Infect. Dis*. 46, 678-685.

Hällgren, A., C. Claesson, B. Saeedi, H.J. Monstein, H. Hanberger, and L.E. Nilsson. 2009. Molecular detection of aggregation substance, enterococcal surface protein, and cytolysin genes and *in vitro* adhesion to urinary catheters of *Enterococcus faecalis* and *E. faecium* of clinical origin. *Int. J. Med. Microbiol.* 299, 323-332.

Hammerum, A.M. and L.B. Jensen. 2002. Prevalence of *esp*, encoding the enterococcal surface protein, in *Enterococcus faecalis* and *Enterococcus faecium* isolates from hospital patients, poultry, and pigs in Denmark. *J. Clin. Microbiol*. 40, 4396.

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

Heikens, E., M. Leendertse, L.M. Wijnands, M. van Luit-Asbroek, M.J. Bonten, T. van der Poll, and R.J. Willems. 2009. Enterococcal surface protein Esp is not essential for cell adhesion and intestinal colonization of *Enterococcus faecium* in mice. *BMC Microbiol*. 29, 9-19.

Henrique, P.M., I.C. Palazzo, R.C. Zanella, and A.L. Darini. 2008. Molecular characterization of enterococci harboring genotype and phenotype incongruence related to glycopeptide resistance isolated in Brazilian hospitals. *Mem. Inst. Oswaldo Cruz* 103, 301-305.

Horan, T.C., M. Andrus, and M.A. Dudeck. 2008. CDC/NHSN surveillance definition of healthcare-associated infection and criteria for specific types of infections in the acute care setting. *Am. J. Infect. Control* 36, 309-332.

Hoshuyama, T., H. Moriguchi, T. Muratani, and T. Matsumoto. 2008. Vancomycin-resistant enterococci (VRE) outbreak at a university hospital in Kitakyushu, Japan: case-control studies. *J. Infect. Chemother.* 14, 354-360.

Izumi, E., P. Domingues Pires, E. Bittencourt de Marques, and S. Suzart. 2005. Hemagglutinating and hemolytic activities of *Enterococcus faecalis* strains isolated from different human clinical sources. *Res. Microbiol.* 156, 583-587.

Jett, B.D., H.G. Jensen, R.E. Nordquist, and M.S. Gilmore. 1992. Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. *Infect. Immun.* 60, 2445-2452.

Kristich, C.J., Y.H. Li, D.G. Cvitkovitch, and G.M. Dunny. 2004. Esp-independent biofilm formation by *Enterococcus faecalis*. *J. Bacteriol*. 186, 154-163.

Leavis, H., J. Top, N. Shankar, K. Borgen, M. Bonten, J. van Embden, and R.J. Willems. 2004. A novel putative enterococcal pathogenicity island linked to *esp* virulence gene of *Enterococcus faecium* and associated with epidemicity. *J. Bacteriol.* 186, 672-682.

Lowe, A.M., P.A. Lambert, and A.W. Smith. 1995. Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with adhesins from some oral streptococci. *Infect. Immun*. 63, 703-706.

Mäkinen, P.L., D.B. Clewell, F. An, and K.K. Mäkinen. 1989. Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from *Streptococcus faecalis* (strain 0G1-10). *J. Biol. Chem*. 264, 3325-3334.

Park, S.Y., K.M. Kim, J.H. Lee, S.J. Seo, and I.H. Lee. 2007. Extracellular gelatinase of *Enterococcus faecalis* destroys a defense system in insect hemolymph and human serum. *Infect. Immun.* 75, 1861-1869.

Petrich, A.K., K.E. Luinstra, D. Groves, M.A. Chernesky, and J.B. Mahony. 1999. Direct detection of *van*A and *van*B genes in clinical specimens for rapid identification of vancomycin resistant enterococci (VRE) using multiplex PCR. *Mol. Cell Probes* 13, 275-

281.

Shankar, N., A.S. Baghdayan, and M.S. Gilmore. 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. *Nature* 417, 746-750.

Shankar, N., C.V. Lockatell, A.S. Baghdayan, C. Drachenberg, M.S. Gilmore, and D.E. Johnson. 2001. Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. *Infect. Immun*. 69, 4366-4372.

Singh, K.V., T.M. Coque, G.M. Weinstock, and B.E. Murray. 1998a. *In vivo* testing of an *Enterococcus faecalis efa*A mutant and use of *efa*A homologs for species identification. *FEMS Immunol. Med. Microbiol*. 21, 323-331.

Singh, K.V., X. Qin, G.M. Weinstock, and B.E. Murray. 1998b. Generation and testing of mutants of *Enterococcus faecalis* in a mouse peritonitis model. *J. Infect. Dis.* 178, 1416-1420.

Sood, S., M. Malhotra, B.K. Das, and A. Kapil. 2008. Enterococcal infections and antimicrobial resistance. *Indian J. Med. Res.* 128, 111-121.

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

Su, Y.A., M.C. Sulavik, P. He, K.K. Mäkinen, P.L. Mäkinen, S. Fiedler, R. Wirth, and D.B. Clewel. 1991. Nucleotide sequence of the gelatinase gene (*gel*E) from *Enterococcus faecalis* subsp. *liquefaciens*. *Infect. Immun*. 59, 415-420.

Tendolkar, P.M., A.S. Baghdayan, M.S. Gilmore, and N. Shankar. 2004. Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis. Infect. Immun*. 72, 6032-6039.

Titze-de-Almeida, R., M. Rollo Filho, C.A. Nogueira, I.P. Rodrigues, J. Eudes Filho, R.S. Nascimento, R.F. Ferreira 2nd, L.M. Moraes, H. Boelens, A. Van Belkum, and M.S. Felipe. 2004. Molecular epidemiology and antimicrobial susceptibility of *enterococci* recovered from Brazilian intensive care units. *Braz. J. Infect. Dis*. 8, 197-205.

Toledo-Arana, A., J. Valle, C. Solano, M.J. Arrizubieta, C. Cucarella, M. Lamata, B. Amorena, J. Leiva, J.R. Penadés, and I. Lasa. 2001. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Appl. Environ. Microbiol*. 67, 4538-4545.

Top, J., R. Willems, and M. Bonten. 2008. Emergence of CC17 *Enterococcus faecium*: from commensal to hospital-adapted pathogen. *FEMS Immunol. Med. Microbiol*. 52, 297-308.

Top, J., R. Willems, S. van der Velden, M. Asbroek, and M. Bonten. 2008. Emergence of clonal complex 17 *Enterococcus faecium* in The Netherlands. *J. Clin. Microbiol.* 46, 214-219.

Treitman, A.N., P.R. Yarnold, J. Warren, and G.A. Noskin. 2005. Emerging incidence of *Enterococcus faecium* among hospital isolates (1993-2002). *J. Clin. Microbiol*. 43, 462-463.

Van Wamel, W.J., A.P. Hendrickx, M.J. Bonten, J. Top, G. Posthuma, and R.J. Willems. 2007. Growth condition-dependent Esp expression by *Enterococcus faecium* affects initial adherence and biofilm formation. *Infect. Immun.* 75, 924-931.

Vankerckhoven, V., G. Huys, M. Vancanneyt, C. Snauwaert, J. Swings, I. Klare, W. Witte, T. Van Autgaerden, S. Chapelle, C. Lammens, and H. Goossens. 2008. Genotypic diversity, antimicrobial resistance, and virulence factors of human isolates and probiotic cultures constituting two intraspecific groups of *Enterococcus faecium* isolates. *Appl. Environ. Microbiol.* 74, 4247-4255.

Vankerckhoven, V., T. Van Autgaerden, C. Vael, C. Lammens, S. Chapelle, R. Rossi, D. Jabes, and H. Goossens. 2004. Development of a multiplex PCR for the detection of *asa*1, *gel*E, *cyl*A, *esp* and *hyl* genes in *enterococci* and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *J. Clin. Microbiol*. 42, 4473-4479.

Waters, C.M., M.H. Antiporta, B.E. Murray, and G.M. Dunny. 2003. Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J. Bacteriol*. 185, 3613-3623.

Werner, G., I. Klare, C. Fleige, and W. Witte. 2008. Increasing rates of vancomycin resistance among *Enterococcus faecium* isolated from German hospitals between 2004 and 2006 are due to wide clonal dissemination of vancomycin-resistant *enterococci* and horizontal spread of *van*A clusters. *Int. J. Med. Microbiol*. 298, 515-527.

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

Willems, R.J., W. Homan, J. Top, M. van Santen-Verheuvel, D. Tribe, X. Manzioros, C. Gaillard, and *et al*. 2001. Variant *esp* gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospital. *Lancet* 357, 853-855.

Worth, L.J., M.A. Slavin, V. Vankerckhoven, H. Goossens, E.A. Grabsch, and K.A. Thursky. 2008. Virulence determinants in vancomycin-resistant *Enterococcus faecium van*B: clonal distribution, prevalence and significance of *esp* and *hyl* in Australian patients with haematological disorders. *J. Hosp. Infect.* 68, 137-144.