Virulence factors in vancomycin-resistant and vancomycin-susceptible Enterococcus faecalis from Brazil

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VIRULENCE FACTORS IN VANCOMYCIN-RESISTANT AND VANCOMYCIN- SUSCEPTIBLE *ENTEROCOCCUS FAECALIS* FROM BRAZIL

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ABSTRACT

Enterococci are members of commensal flora of animals and insects, but are also important opportunistic pathogens. Our objective was to observe if there was any difference of virulence in several groups of *E. faecalis,* mainly between vancomycin-resistant *E. faecalis* (VREFS) of colonization and infection. VREFS and vancomycin-sensitive *E. faecalis* from Brazil were screened for the presence of virulence factor genes. Phenotypic assays were used to assess in vitro expression, to understand the pathogenic potential of these isolates and to determine whether a correlation exists between virulence and antibiotic resistance. Different virulence profiles were found suggesting that the disseminating clone may have generated several variations. However, our study showed that one constellation of traits appeared most commonly: gelatinase, aggregation substance and *esp* (GEA). These factors are important because they have been implicated in cell aggregation and biofilm formation. Biofilm formation may promote the conjugation of plasmids harboring resistance and virulence genes, enhancing the probability of entry of new resistance genes into species. Curiously, the profile GEA was not exclusive to VREFS, it was the second most observed in VSEFS isolates from colonization and infection in hospitalized patients and also from rectal swabs of healthy volunteers. Such strains appear to represent the entry gateway to new resistance genes into *E. faecalis* and may contribute to the spreading of *E. faecalis* mainly in hospitals.

Key-words: *Enterococcus faecalis*, VRE, Esp, gelatinase, aggregation substance

INTRODUCTION

Enterococci are members of the commensal flora of human, animals and insects, but are also important opportunistic pathogens that often posses several vitulence factors and resistance to multiple antibiotics including vancomycin (4). Of the five phenotypes of glycopeptide resistance described (4,11,26) VanA and VanB are the most commom (26).

Cytolysin (Cyl), enterococcal surface protein (Esp), gelatinase (GelE) and aggregation substance (Agg) are among the virulence factors able to influence host/parasite relationships that were described in *Enterococcus faecalis* (7,15,21). Cytolysin is a hemolytic toxin that also has bacteriocin activity (7). Esp is a surface protein that enhances the biofilm formation by *E. faecalis* (23). Gelatinase is a metalloprotease that cleaves several substrates including insoluble collagen fragments, as well as the pheromone and inhibitor peptides involved in conjugative plasmid transfer of *E. faecalis* (25). A role for GelE in biofilm development has also been recently described (14,15). Aggregation substance, encoded by *asc 10, asa1* and other related genes, is a pheromone-inducible surface protein that promotes aggregation during bacterial conjugation in *E. faecalis* (5). Some of these factors, including cytolysin, Agg and Esp were found to be encoded on a large, 153-kb pathogenicity island (20). These virulence factors may play a role in promoting persistence of enterococci in the nosocomial

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environment, and consequently inter and intra-hospitals dissemination.

In Brazil, vancomycin resistant Enterococci (VRE) have been isolated only from hospitalized patients (27) and have not yet been described in animals despite the past use of avoparcin as growth promoter (16). The first VRE strain in Brazil was isolated in 1996 and was *E. faecium* classified as VanD-4 and ST281 by MLST (1,10,11). However, no other VanD strains have been reported in Brazil. On the other hand, a predominant clone of vancomycin-resistant *E. faecalis* (VREFS) VanA has recently been disseminating throughout hospitals in Sao Paulo and other cities (8,18,19). It was of interest to identify virulence factors and antibiotic resistance profiles among isolates of the disseminating clone and compare them with subsets of vancomycin-sensitive *E. faecalis* (VSEFS) from several sources in Brazil. In addition, to know the isolate pathogenicities was the base for understanding if a correlation exists between virulence factors and resistance and to determine whether the dissemination of this *E. faecalis* clone represents an important shift in the spread of vancomycin resistance to more virulent lineages.

MATERIAL AND METHODS

A total of 115 isolates of *E. faecalis* from several regions of Brazil, were divided into two major groups, VREFS (24 isolates) and VSEFS (91 isolates). VREFS isolates were recovered only from hospitalized patients in Brazil, and were subdivided in 2 subsets: 8 VREFS from colonized hospitalized patients (chp) and 16 VREFS from infections in hospitalized patients (ihp). The 91 VSEFS were subdivided into 3 different subsets: 41 VSEFS ihp, 34 VSEFS chp and 16 VSEFS from colonized volunteers within the community (cvc). Species and genotype identification of VRE were performed by PCR as previously described (13). Tests for high-level gentamicin (500 µg/mL) and streptomycin (2,000mg/mL) and Minimal Inhibitory Concentrations (MICs) of vancomycin and penicillin were determined by the agar dilution method as recommended by CLSI (6).

Total DNA was prepared as described (21), and 200 ng of each DNA sample was bound to a zeta-probe nylon membrane (Bio Rad, Hercules, California) using a 96 wells microfiltration apparatus (Bio Rad, Bio-Dot®). The DNA was crosslinked to the membrane using an ultraviolet multilinker (UVC 515, Ultra-Lum, Carson, California, USA), and hybridized to radioactive probes. Genes of interest were amplified by PCR using the primers described in Table 1. The probes were labeled using the *RadPrime DNA Labeling System* (Invitrogen, California, USA).

Gelatinase production was determined on Todd-Hewitt agar supplemented with 3% gelatin (22). Cytolysin production was determined by observing β-hemolysis on BHI agar supplemented with 5% (v/v) human erythrocytes. Control strains included: *E. faecalis* JH2SS (pAD1) for the *asa1* gene; *E. faecalis* MMH594 for the genes *asc10*, *cylL*$_L$, *cylL*$_S$, *cylA* and *esp*; *E. faecalis* OG1RF for the *gelE* gene.

Agg expression was detected by the clumping assay (12) with pheromones obtained from *E. faecalis* FA2-2. To precipitate pheromones, an overnight 1:10 culture of plasmid-free *E. faecalis* FA2-2 was subcultured into 200 mL of THB for 6 h at 37ºC. The cells were removed by centrifugation, and the supernatant was filtered through a 0.2 µm filter (Nalgen®, Nalge

Table 1. Primers used to make the specific probes for hybridization.

Gene	Primer name	Sequence 5'-3'	Lenght of the fragment	Reference
cylL$_L$	*cylL*$_L$ F	AAC TAA GTG TTG AGG AAA TG	159bp	(2)
	cylL$_L$*R*	*AAA GAC ACA ACT ACA GTT AC*		
*cylL*s	*cylL*s F	AGA ACTTGT TGG TCC TTC	134 bp	(2)
	*cylL*s R	GCT GAA AAT AAT GCA CCT AC		
*cyl*A	*cyl*A F	ACA GGT TAT GCA TCA GAT CT	507bp	(2)
	*cyl*A R	AAT TCA CTC TTG GAG CAA TC		
*esp***	Esp14F	AGA TTT CAT CTT TGA TTC TTG G	500 bp	AF034779*
	Esp12R	AAT TGA TTC TTT AGC ATC TGG		
*gel*E	*gel*E F	AATTGCTTTACACGGAACGG	548bp	(2)
	*gel*E R	GAGCCATGGTTTCTGGTTGT		
Agg 1*** (*asa*1 and *asc*10)	Agg 1 F	AGT GAC GAT TGA TTTATC CAA AGT G	446bp	X17214 and M64978*
	Agg 1 R	CGT TCG GAA GAT TTC ACT TCA TAA T		

* The primers were designed based on the sequences registered in the GenBank. ** The sequences of *esp* primers were provided by Dr. Nathan Shankar, College of Pharmacy, Oklahoma University Health Sciences Center, OK, USA. *** Primers are the same for amplify both genes, but the templates were different.

Nunc International, Rochester, NY). Pheromones were precipitated from supernatant with trichloroacetic acid at a final concentration of 5% for 18h at 4ºC. Precipitate was collected by centrifugation at 8410 g for 15 minutes, dried and dissolved in 10 mL of Todd Hewitt Broth (THB), and again filtered.

All VRE isolates were typed by pulsed-field gel electrophoresis (PFGE) as described (3). Restriction fragments were separated using Gene Navigator apparatus (Amersham Biosciences) at 180 V for 25 h, at 7ºC, adjusted for pulse times of 20 s for 10 h, 8 s for 10 h and 3 s for 5 h. PFGE clonal groups were assigned according to the criteria of Tenover *et al.* (24). The project protocol was approved by the Research Ethical Committee of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, under the number 28/CEP/FCFRP.

Statistical analysis was performed by Fisher's exact test using GraphPad Prism software (San Diego, California, USA).

RESULTS AND DISCUSSION

PFGE typing of all VREFS in this study confirmed the presence of the VREFS type A strain in several hospitals in different cities of Brazil (Table 2 and Fig.1). All but one VREFS were resistant to penicillin and high levels of gentamicin, but susceptible to streptomycin. The remaining PFGE types, resistance and virulence profiles are shown in Table 2.

Virulence factor profiles were established by determining the presence of the gene and confirming its expression in the phenotypic assays, except for *esp*. This study has shown that PFGE type A strain appears to have undergone small variations with time, since several virulence profiles were found in different isolates of the same strain. Among the varied virulence profiles, one was the most common among isolates of this disseminating clone. Twelve out 16 type A isolates possessed the GelE, Agg and *esp* profile (Table 2). Our results suggest that loss of some factors resulted in the variations found in the 4 remaining type A isolates.

Among the VREFS, cytolysin was presented in only one isolate that also produced gelatinase. This isolate was a subtype A1 strain, closely related to the disseminating clone in Brazil. However, another VREFS subtype A1, isolated 2 years later, possessed the GelE, Agg and *esp* profile. The data suggests that either the first isolate acquired the cytolysin operon,

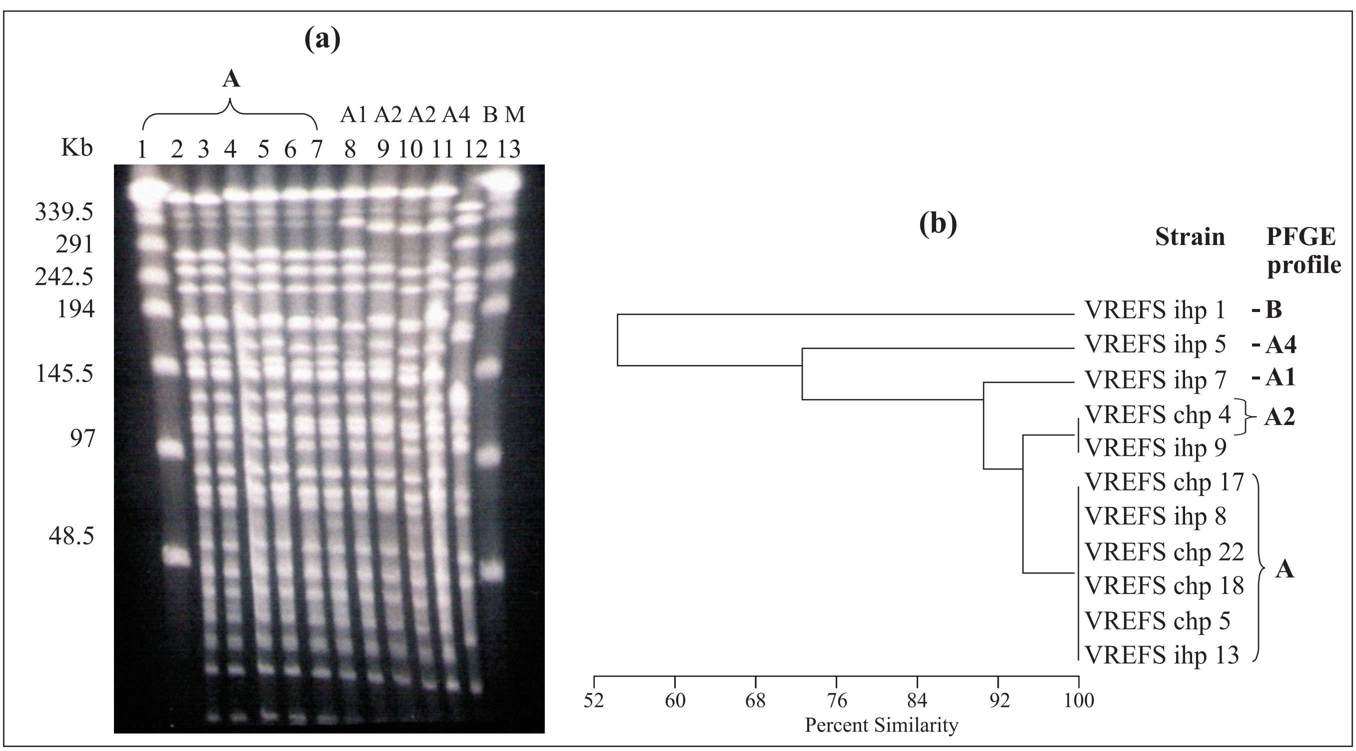

Figure 1. Agarose gel electrophoresis of genomic DNA. a) Results of pulsed-field Gel electrophoresis analysis of VREFS isolated in Brazil. 1- M, Lambda PFMarker (New England BioLabs), 2- VREFS ihp 13, 3- VREFS chp 5, 4- VREFS chp 18, 5- VREFS chp 22, 6- VREFS ihp 8, 7- VREFS chp 17, 8- VREFS ihp 7, 9- VREFS ihp 9, 10- VREFS chp 4, 11- VREFS ihp 25, 12- VREFS ihp 1, 13- M Lambda PFMarker (New England BioLabs); b) Dendrogram of PFGE of the strains calculated with percent similarity performed by MVSP 3.0 software. chp, isolates of hospitalized and colonized patients; ihp, isolates from infection in hospitalized patients.

Table 2. Date, susceptibility profile, virulence factors and PFGE profile present in vancomycin-resistant *E. faecalis* isolates from Brazil.

Samples	**Isolation**		**Virulence factors**	**Susceptibility profile**	**PFGE profile**
	Date	**Source** [a]	**Genes** [b] **/ Expression profile** [c]	**P /HLAR G /HLAR S** [d]	
VREFS chp [e]					
4	1998	CSSM-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A2
5	1998	CSSM-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
6	1998	CSSM-SP	*asa1, asc10 esp* / AE	R/R/S	A
18	1999	HCSP-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
22	2000	HCASP-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
24	1998	CSSM-SP	*gelE, asa1, asc10 esp* / AE	R/R/S	C
17	1999	CSSM-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
23	1999	HSPSP-SP	*gelE, asa1, asc10 esp* / GAE	R/S/S	A
VREFS ihp [f]					
1	1998	CSSM-SP	*gelE, asa1, asc10* / N	R/R/S	B
2	1998	CSSM-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
3	1998	CSSM-SP	*gelE,* $cylL_LL_SA$*, asa1, asc10* / GC	R/R/S	A1
7	2000	HEDPA - RS	*gelE, asa1, asc10 esp* / GAE	R/R/S	A1
8	2000	HPUCP-RS	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
9	2000	HCC-PR	*gelE, asa1, asc10 esp* / GAE	R/R/S	A2
10	2000	HCC-PR	*gelE, asa1, asc10 esp* / GAE	R/R/S	A3
11	1999	CSSM-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
12	1999	CSSM-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
13	1999	CSSM-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
14	1999	CSSM-SP	*gelE, asa1, asc10* / GA	R/R/S	A
15	1999	CSSM-SP	*gelE, asa1, esp* / GAE	R/R/S	A
16	1999	CSSM-SP	*gelE, asa1, asc10 esp* / GAE	R/R/S	A
20	2000	CSSM-SP	*gelE, asa1, asc10 esp* / GE	R/R/S	A
21	1999	HSPSP-SP	*gelE, asa1, asc10 esp* / G	R/R/S	A
25	2003	UFU - MG	*gelE, asa1, asc10 esp* / GAE	R/R/S	A4

[a] CSSM-SP – Casa de Saúde Santa Marcelina – SP, HCSP-SP- Hospital do Coração de São Paulo, HSPSP-SP – Hospital do Servidor Público de São Paulo, HED PA – RS– Hospital Ernesto Dornelles - Porto Alegre - RS, HPUCPA – RS- Hospital da Pontifícia Universidade Católica de Porto Alegre - RS, HCC-PR – Hospital das Clínicas de Curitiba - PR, HCASP-SP – Hospital Cruz Azul – SP, UFU – MG, Universidade Federal de Uberlandia. [b] *gelE,* gelatinase gene; $cylL_LL_SA$, cytolysin genes; *asa1* and *asc10* are aggregation substance's genes; *esp,* enterococcal surfae protein gene. [c] G, gelatinase, A, aggregation, E, *esp* presence, C, cytolysin activity, N, none of the virulence factors studied found were expressed. [d] P, penicillin, HLAR G, high level aminoglycoside resistance - gentamycin, HLAR S, high level Aminoglycoside resistance - streptomycin, R, resistant and S, susceptible. [e] chp, isolates of hospitalized colonization patients. [f] ihp, isolates of infection in hospitalized patients.

perhaps on a conjugative plasmid, but lost *esp,* or had not acquired it yet. In addition, both Agg genes were detected in the strain, but not their expression. In our study, the total incidence of cytolysin among VSEFS isolates was 12% (11 out of 91), in concordance with reported observations (9).

The presence of the gelatinase gene by itself, for instance, does not ensure its expression. The expression of virulence factor's gene may be controlled by a very complex regulation network and is not the focus of this study. On the other hand, some VSEFS isolates were positive for clumping but lacked the two aggregation substance genes studied, indicating that other Agg genes may be involved in the aggregation by some VSEFS. This was especially true in the VSEFS cvc group, among which 31.2% were positive by phenotypic assay, but negative for *asc10* and *asa1*. Strains with *asa1* and *asc10* genes*,* but negative in phenotypic assays, were also found suggesting that the presence of these genes may not predict their expression. In summary, *asa1* and *asc10* are present and expressed in a large number of VREFS isolates, but greater variability exists in VSEFS isolates.

Subtypes A2, A3 and A4 were isolated in different periods of time, but presented the same GelE, Agg and *esp* virulence profile. The only VREFS type C strain in this study showed the

Agg-*esp* profile and had a non-expressed *gelE* gene. Finally, there was only one VREFS type B in this study that in spite of having *gelE* and Agg genes did not express them. Therefore, it did not fit any of the virulence factor profiles. Only one of the VSEFS strains (chp group) did not possess any of the virulence factor genes tested but another VSEFS (ihp group) possessed all. There was no absolute correlation between the presence of particular virulence factors and resistance to the antibiotics in the VSEFS isolates (data not shown). Factors GelE, Agg, Cyl and *esp* were detected in isolate VSEFS iph JF7 which was sensitive to all antibiotics. Resistance to penicillin was observed in all VREFS, most were also resistant to high level gentamicin but all were sensitive to high level streptomycin (Table 2)

Marques *et al.* (17) described that there was no significant association between virulence factor markers and clinical sources in a different set of strains isolated in Brazil. The most frequent genotypic profile detected by Marques *et al*. (17) was *efa* (adhesin), *esp* and *gelE*. We did not search for *efa* in our work, but in both VREFS and VSEFS groups, most isolates exhibited the following profiles: Agg and *esp*, or GelE, Agg and *esp*. All VSEFS subsets showed profiles as, *esp* only, gelatinase only, and Cyl, Agg and *esp*. Profiles GelE and Cyl, and GelE and Agg were detected among VSEFS from colonized volunteers of the community, and from infections in hospitalized patient. Agg occurring by itself was found only in VSEFS from colonization subsets.

In conclusion, there was no difference statistically significant in the frequency of the virulence factor's genes found in the type A strains (Table 2) when compared both groups of VREFS (chp and ihp) (Fisher's exact test, $p = 0.59$). The virulence factors GelE, Agg and *esp* occur together in most VREFS type A isolates, the disseminating clone occurring in Brazil. However, this constellation of traits is not exclusive of VREFS but it was also observed in VSEFS isolates of colonization and infection patients as well as of healthy colonization volunteers. These factors have all been shown by other investigators to aggregate cells, to contribute to biofilm formation and to be particularly important in the dissemination or acquisition of resistances by promoting cell-cell contact and the conjugal transfer of plasmids harboring resistance and virulence genes. Such strains appear to represent the entry gateway to new resistance genes into *E. faecalis* and may contribute to the spreading of *E. faecalis* mainly in the hospitals.

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RESUMO

Fatores de virulência em *Enterococcus faecalis* resistentes e suscetível à vancomicina isolados no Brasil

Enterococci são membros da microbiota comensal de animais e insetos, mas também são importantes patógenos oportunistas. Nosso objetivo foi observar se há qualquer diferença na virulência nos diversos grupos de *Enterococcus faecalis*, principalmente nos *E. faecalis* resistente à vancomicina (VREFS) isolados de colonização e infecção. VREFS e *E. faecalis* sensíveis à vancomicina (VSEFS) do Brasil foram pesquisadas quanto a presença de fatores de virulência. Ensaios fenotípicos foram usados para obter a expressão in vivo, entender o potencial patogênico destas amostras e determinar se existe correlação entre virulência e resistência a antibióticos. Diferentes perfis de virulência foram encontrados sugerindo que o clone que está se disseminado pode ter gerado diversas variações. No entanto, nosso estudo mostrou que um conjunto de fatores parece ser mais comum entre as amostras: gelatinase, substância de agregação e *esp* (GEA). Estes fatores tem sido correlacionados com a agregação de células e formação de biofilmes. A formação de biofilme pode promover a conjugação de plasmídeos contendo genes de resistência entre as espécies. Curiosamente, o perfil GAE não foi exclusivo para VREFS, foi o segundo mais observado em amostras VSEFS provenientes de colonização e infecção em pacientes hospitalizados e também de *swabs* retais de voluntários saudáveis. Tais linhagens pacerem representar a "porta de entrada" para novos genes de resistência em *E. faecalis* e podem contribuir para a disseminação de *E. faecalis* principalmente nos hospitais.

Palavras-chave: *Enterococcus faecalis*, VRE, Esp, gelatinase, substância de agregação

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