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Research Paper

Molecular detection of virulence factors among food and clinical *Enterococcus faecalis* strains in South Brazil

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Abstract

The present report aimed to perform a molecular epidemiological survey by investigating the presence of virulence factors in *E. faecalis* isolated from different human clinical (n = 57) and food samples (n = 55) in Porto Alegre, Brazil, collected from 2006 to 2009. In addition, the ability to form biofilm *in vitro on* polystyrene and the β-haemolytic and gelatinase activities were determined. Clinical strains presented a higher prevalence of aggregation substance (*agg*), enterococcal surface protein (*esp*) and cytolysin (*cylA*) genes when compared with food isolates. The *esp* gene was found only in clinical strains. On the other hand, the gelatinase (*gel*E*)* and adherence factor (*ace*) genes had similar prevalence among the strains, showing the widespread occurrence of these virulence factors among food and clinical *E. faecalis* strains in South Brazil. More than three virulence factor genes were detected in 77.2% and 18.2% of clinical and food strains, respectively. Gelatinase and β-haemolysin activities were not associated with the presence of *gel*E and *cylA* genes. The ability to produce biofilm was detected in 100% of clinical and 94.6% of food isolates, and clinical strains were more able to form biofilm than the food isolates (Student's *t*-test, $p < 0.01$). Results from the statistical analysis showed significant associations between strong biofilm formation and *ace* ($p = 0.015$) and *gelE* ($p = 0.007$) genes in clinical strains. In conclusion, our data indicate that *E. faecalis* strains isolated from clinical and food samples possess distinctive patterns of virulence factors, with a larger number of genes that encode virulence factors detected in clinical strains.

Key words: virulence determinants, clinical enterococci, food enterococci, biofilm formation.

Introduction

Enterococcus faecalis are common bacteria that inhabit the gastrointestinal tract of humans and animals, being widely distributed in soil, water, plants and food products (Giraffa 2006; Frazzon *et al.*, 2009). These microorganisms have been associated with nosocomial infections, bacteremia, surgical wound infection, endocarditis, and urinary tract infections (Huycke *et al.*, 1998; Samuelsson *et al.*, 2003; d'Azevedo *et al.*, 2006). The enterococcal infection is complicated by the intrinsic resistance to some antimicrobials commonly prescribed for Gram-positive cocci, such as cephalosporin, lincomycin, cotrimoxazole, and low levels of penicillin and aminoglycosides, as well as by the ability to acquire resistance genes via transposons or plasmids (Huycke *et al.*, 1998; Kayser 2003).

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Virulence factors of *E. faecalis* are thought to ease adherence of bacteria to the host cell membranes and to environmental surfaces, where they can obtain nutrients and evade the host immune response. The extracellular surface protein (Esp), encoded by the *esp* gene, is a cell wall-associated protein which serve as adhesin for the pathogen to host tissue colonization and persistence in urinary tract infections (Fischer and Phillips, 2009). Another cell surface protein present in *E. faecalis* is adhesion of collagen (Ace), which mediates the association of bacteria to host cell matrix proteins, such as collagen I and IV and laminin. Studies have demonstrated that Ace protein may contribute to the pathogenesis of enterococci in endocarditis (Koch *et al.*, 2004; Lebreton *et al.*, 2009; Upadhyaya *et al.*, 2009). The aggregation substance (Agg) is a pheromone-inducible surface protein of *E. faecalis* required for cell-to-cell contact during conjugation and for adhesion to eukaryotic cells. This protein eases aggregation of donor and recipient bacteria and aids conjugative plasmid transfer during bacterial conjugation. Studies have revealed that aggregation substance may promote adherence of bacterial proteins to extracellular matrix proteins and also may increase cell surface hydrophobicity (Cariolato *et al.*, 2008; Upadhyaya *et al.*, 2009).

Some extracellular proteins, such as gelatinase and cytolysin, have been classified as virulence factors of enterococci. Gelatinase is a zinc metalloprotease encoded by the chromosomal *gel*E gene. It hydrolyzes gelatin, casein, hemoglobin, and other bioactive compounds (Walters *et al.*, 2003). The cytolysin, a bacterial toxin with β-haemolytic properties in humans and with bacteriocin activity against other Gram-positive bacteria, is encoded by the cytolysin operon (*cylR1*, cylR2, $cylL_L$, $cylL_S$, *cylM*, *cylB*, *cylA*, *cyl*I). The proteins encoded by $cylL_L$ and $cylL_S$ are precursors of two small lantibiotic-like peptides, which are modified by the product of *cylM* gene. The peptides are transported out of the cell by the product of *cylB* gene, an ATP-binding cassette transporter. The *cylA* gene product processes the peptides and activates them, allowing them to combine and then to produce the cytolysin. The mechanism that signals the upregulation of the operon involves two regulator proteins corresponding to the *cylR1* and *cylR2* gene products from a divergently transcribed operon. An immunity function is present and is provided by a membrane protein encoded by the *cylI* gene (Gaspar *et al.*, 2009; Singh *et al.*, 2010).

The ambiguous role of *Enterococcus* has stimulated research in order to determine particular characteristics of enterococci isolated from food and clinical samples and to solve the questions about the risks of using this microorganism in food. In addition, the pathogenesis of these microorganisms remains unclear, but it is proposed that some virulence factors could increase the capacity of *Enterococcus* to cause infection, facilitating the adhesion and colonization (Franz *et al.*, 2003). So far, in Brazil, there are no studies investigating the prevalence of virulence factors among food and clinical strains of *E. faecalis*. This study aimed to investigate the prevalence of virulence factors in *E. faecalis* isolated from diverse human clinical and food samples collected in South Brazil, as well as to access their ability to form biofilm *in vitro* on polystyrene and to determine their β-haemolytic and gelatinase activities.

Materials and Methods

Enterococcus faecalis isolates

The *E. faecalis* investigated in the present study were isolated from different human clinical samples (urine, blood and cervical secretion) (n = 57) and foods samples (cassava, beetroot, potato, sweet potato, parsley, cabbage, raw meat, pasteurized milk and dairy products, such colonial cheese type and soft cheese) (n = 55) in Porto Alegre, Brazil, from 2006 to 2009 (d'Azevedo *et al.*, 2006, Riboldi *et al.*, 2009). All strains had already been identified to genus level by PCR with genus-specific primer pairs and to species level using the conventional biochemical tests (Dutka-Mahlen *et al.*, 1995; Facklam *et al.*, 2002).

Detection of virulence factors genes by PCR method

The virulence factors genes *gelE* (gelatinase), *esp* (extracellular surface protein), *agg* (aggregation substance), *ace* (adhesin of collagen) and *cylA* (activator of cytolysin) were identified by PCR, using specific primes (Duprè *et al.*, 2003). *E.faecalis* JH2-2 and MMH594 were used as positive controls.

Detection of β-haemolytic and gelatinase activities

The β-haemolytic activity was determined by streaking the strains on Columbia Agar (Himedia Laboratories Ltd., India) supplemented with 5% defibrinated horse blood (Semedo *et al.*, 2003). The gelatinase activity was performed in tubes containing brain heart infuse on broth (BHI) supplemented with 12% of gelatin substrate (Himedia Laboratories Ltd., India) (Marra *et al.*, 2007).

Biofilm formation in vitro assay

Biofilm formation on polystyrene microplates was quantified by the crystal violet staining method with some improvements (Stepanovic *et al.*, 2000). Briefly, bacteria were cultured at 36°C in tryptic soy broth (TSB) for 18 h. The wells of sterile 96-well flat-bottomed polystyrene microplates were filled with 180 μL of TSB supplemented with 0.75% glucose (TSBG) and 20 μL of bacterial suspension containing approximately 10^8 cfu/mL. Subsequently, the plates were incubated for 18 h at 36 °C. The optical density (O.D.) was measured at 492 nm ($O.D._{492}$) in spectrophotometer (Anthos 2010 Microplates Reader) (Vogel *et al.*, 2000). The cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of the negative control. All strains were separated into categories using the O.D. measurement of bacterial films,

as it follows: O.D. ≤ O.D.c= non-adherent, O.D.c < O.D. ≤ (2x O.D.c) = weakly adherent, (2x O.D.c) < O.D. ≤ (4x O.D.c) = moderately adherent and (4x O.D.c) < O.D. = strongly adherent (Christensen *et al.*, 1985). The experiments were performed at least three times for each strain. *Staphylococcus* epidermidis American Type Culture Collection 35984 classified as a strong adherent was used as the positive control. This strain was selected because it has been used successfully in research of biofilm (Hufnagel *et al.*, 2004; Marinho *et al.*, 2013).

Statistical analysis

The results were analyzed using the Chi-square test and Fischer's exact, followed by residual analysis. All statistical analyses were performed with Statistic Package of the Social Science software (SPSS) 13th edition and a p value < 0.05 was considered significant. Student's *t*-test was used to compare the absorbance values of biofilm formers among clinical and food isolates. Results were also considered significant when p value was ≤ 0.05.

Results

Prevalence of virulence genes among clinical and food enterococci

The distributions of virulence factor genes among clinical and food *E. faecalis* are show in Table 1. Clinical strains displayed the highest prevalence of aggregation substance (agg), enterococcal surface protein (*esp*) and cytolysin (c*ylA*) genes when compared with food isolates, and, in addition, *esp* gene was found only in clinical strains. On the other hand, the gelatinase (*gel*E*)* and adherence factor (ace) genes had similar prevalence among the strains, showing the widespread occurrence of these virulence factors among food and clinical *E. faecalis* strains in South Brazil.

In a total of 112 *E. faecalis*, the *gel*E gene was the most prevalent factor (77.7%), followed by the *ace* (74.1%), while *agg*, *esp* and *cylA* genes were detected in 38.4%, 34.8% and 31.25% of the isolates, respectively. Forty-four (77.2%) clinical and 10 (18.2%) food strains harbored more than three virulence factor genes (Figure 1).

Relationship between virulence factor gene and corresponding phenotype

Expression of gelatinase and β-haemolysin *in vitro* was not always correlated with the detection of *gel*E and *cylA* genes by PCR (Table 1). Analysis of congruence between gelatinase and *gel*E pointed out that 24 (54.5%) clinical and 6 (13.9%) *gel*E-positive food strains were unable to degrade gelatin, and non-haemolytic activity was detected in 23 (74.2%) clinical and two (50%) *cylA*-positives food strains.

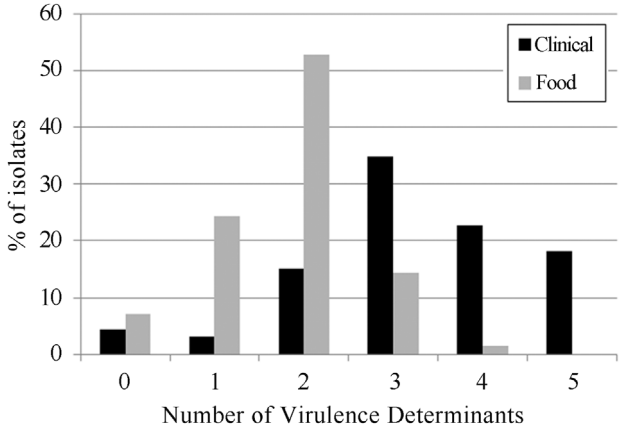

Figure 1 - Distribution of virulence factors among *Enterococcus faecalis* isolated from clinical and food samples.

Biofilm formation and virulence factor genes

Table 2 shows the results of the biofilm formation assay of clinical and food *E. faecalis* strains. All clinical isolates formed biofilm *vs.* 94.5% of food strains, and 49 of 57 clinical strains (86%) were strong biofilm formers *vs.* 31 (56.4%) of the food isolates Statistically significant difference between the amount of biofilm formed by the clinical strains and the one formed by food strains was observed (p < 0.001) Results from the statistical analysis showed significant associations between strong biofilm formation and *ace* (p = 0.015) and *gelE* (p = 0.007) genes in clinical strains. On the other hand, no correlation between biofilm formation and the presence of *agg* (p = 0.115) and *esp* (p = 0.064) genes were observed in clinical *E. faecalis.*

Discussion

Differences in the distribution of virulence factors among *E. faecalis* isolated from clinical samples and food in South Brazil were observed, in which clinical strains exhibited more virulence factors determinants when compared with food isolates. This result agrees with Abriouel *et*

Table 1 - Distribution of virulence genes among *Enterococcus faecalis* isolated from clinical and food samples in South Brazil.

Source	No. (%) of isolates positive to:				
	ace	*esp*	*agg*	*gelE*	*cylA*
Food (*n* = 55)	41 (74.5)	0 (0)	10 (18.2)	43 (78.2)	4 (7.2)
Clinical (*n* = 57)	42 (73.7)	39 (68.4)	33 (57.9)	44 (77.2)	31 (54.4)
Total (*n* = 112)	83 (74.1)	39 (34.8)	43 (38.4)	87 (77.7)	35 (31.5)

Table 2 - Distribution of enterococcal clinical and food *Enterococcus faecalis* strains according to the ability of biofilm formation on polystyrene microtiter plates.

Source	No. (%) of biofilm forming isolates			
	SA	MA	WA	NA
Food (n = 55)	31 (56.4)	15 (27.3)	6 (10.9)	3 (5.5)
Clinical (n = 57)	49 (86)*	5(8.8)*	3 (5.3)*	0 (0)
Total	80 (71.4)	20 (17.9)	9 (8.0)	3 (2.7)

SA: strong adherence; MA: moderate adherence; WA: weak adherence; NA: no adherence. Asterisks denote statistical significance of biofilm formation for clinical strains ($p < 0.01$).

al. (2008) and Cariolato *et al.* (2008), who observed that *E. faecalis* isolated from vegetables and environment harbored fewer determinants when compared with clinical isolates.

Our results showed a high prevalence (57.9%) of *agg* gene in clinical strains. Dupont *et al.* (2008) and Bittencourt and Suzart (2004) also found a similar prevalence of *agg* gene in *E. faecalis* isolated from clinical samples. On the other hand, the prevalence of *agg* (18.2%) in food strains was lower than previously reported by Eaton and Gasson (2001), who encountered a high frequency (67%) of agg in *E. faecalis* strains isolated from food. The difference observed between the previous study and results showed here may be caused by the lower number (n = 8) of *E. faecalis* tested by Eaton and Gasson (2001). Strains harboring *agg* gene should be clearly undesirable in food, since the product of this gene mediates contact between bacterial cells and host cells, facilitating the acquisition of genes and the colonization ability. No correlation between biofilm formation *in vitro* on polystyrene microplates and the presence of agg gene in *E. faecalis* isolated from food and clinical samples was observed in the present study. Aggregation substance plays an important role in mediating efficiently the adhesion between bacteria, thereby easing plasmid exchange. Bacterial adherence appears to be a multifactorial phenomenon involving specific and nonspecific determinants. Studies have shown the relation between adherence or biofilm formation and *E. faecalis* aggregation substance using renal (VERO) and intestinal (CACO-2) epithelial cells or *ex vivo* model of cardiac valve colonization, rather than polystyrene microplates (Duprè *et al.*, 2003; Chuang-Smith *et al.*, 2010).

The occurrence of *esp* only in clinical isolates observed in our study agrees with results obtained by Mannu *et al.* (2003), which revealed the presence of enterococcal surface protein gene only in clinical strains, although they contrast with previous studies that detected *esp* gene in isolates from vegetables and water, possibly reflecting differences in the origins of the isolates (Eaton and Gasson 2001; Drahovská *et al.*, 2004; Abriouel *et al.*, 2008). The role of Esp in enterococcal infection is still undetermined, although most of the *esp*-positive clinical strains were isolated from urinary infection, supporting the function of this protein in tissue colonization (Shankar *et al.*, 2001; Sava *et al.*, 2010). Even though some studies suggest an important role for Esp in enterococcal biofilm formation, in our study, the occurrence of *esp* gene was not correlated with biofilm formation on polystyrene surface. These results are in accordance with previous findings, which have also demonstrated biofilm formation by *esp*-negative *E. faecalis* strains (Duprè *et al.*, 2003; Rosa *et al.*, 2006).

A higher frequency of *cylA* gene in clinical isolates and a lower one in food strains has also been observed in other studies (Semedo *et al.*, 2003; Drahovská *et al.*, 2004). Analysis of congruence between hemolytic activity and isolates carrying the *cylA* gene showed a lack of hemolytic activity in 74.2% of clinical and 50% of *cylA*-positive food isolates. Cosentino *et al.* (2010) have found clinical strains of enterococci carrying the *cly* genes and being non-haemolitic. The lack of cytolysin phenotypic/genotypic congruence may suggest missing genes in the *cyl* operon among *cylA*-positive/haemolysin-negative strains or the presence of silent *cylA* gene (Gaspar *et al.*, 2009; Upadhyaya *et al.*, 2009).

In the present study, an equivalent frequency of *gel*E gene was observed in food and clinical strains. These results are in agreement with data reported by others studies (Mohamed *et al.*, 2004; Abriouel *et al.*, 2008). The presence of the *gelE* gene and the lack of gelatinase activity in *Enterococcus* have been associated with manipulation in laboratory, silent genes, and low level expression or downregulation of the *gelE* gene (Lopes *et al.*, 2006). Our data showed that strong biofilm formation correlated with *gelE* ($p < 0.05$) in clinical strains. Hancock and Perego (2004) and Mohamed *et al.* (2004) encountered a positive relation between *gelE* and biofilm formation. The *gelE* gene might directly or indirectly degrade collagen protein or other tissue proteins of host cells, which may further aggravate tissue injury.

The *ace* gene was distributed equally among clinical and food enterococci. Cariolato *et al.* (2008) reported similar prevalence of *ace* gene in enterococci isolated from human samples and dairy products. A positive association was observed between strong biofilm formation and the presence of *ace* in clinical strains ($p < 0.05$). Our results are in accordance with Singh *et al.* (2010), who observed that deletions in the *ace* gene result in a significant attenuation of the ability of *E. faecalis* to colonize and to cause aortic valve endocarditis, and they showed that Ace plays an important role in the early stages of colonization, possibly by mediating the adherence of *E. faecalis* to collagen exposed at the site of vascular injury. The function of *ace* in *Enterococcus* from other sources has not yet been elucidated.

Almost all isolates were able to form biofilms *in vitro* by the technique of adhesion in polyethylene. The correlation between virulence factor genes and biofilm formation

is contentious. Some researchers affirm that the biofilm formation is a common characteristic of *Enterococccus* (Mohamed *et al.*, 2004; Rosa *et al.*, 2006). Enterococci isolated from food showing ability to form biofilms are alarming, since this ability contributes to survival, persistence and dissemination of resistant enterococci and/or resistance genes in diverse environmental conditions. Moreover, they are potential sources of contamination that might lead to food spoilage or transmission of diseases (Franz *et al.*, 2003). In clinical strains, this ability is also remarkable, since biofilms can be formed in medical devices, such as catheter and surgical prostheses, and infections caused by biofilms are often treated by the implant removal, which increases the trauma and the treatment cost (Baldassarri *et al.*, 2001; Rosa *et al.*, 2006).

In conclusion, our data indicate that *E. faecalis* strains isolated from different clinical samples and foods displays distinctive patterns of virulence factors, since a larger number of genes that encode them were detected predominantly in clinical strains. Silent *cylA* and *gelE* genes were frequently detect in both groups. Despite the fact that E. faecalis isolated from food showed a low frequency of virulence genes, they may act as reservoirs of virulence factors, enabling the dissemination of these genes to the human microbiota through the food chain. Additional investigations are needed to evaluate the expression of such factors, which may not be revealed by *in vitro* phenotypic tests during the course of infection.

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