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Characterization of virulence factors and clonal diversity of *Enterococcus faecalis* isolates from treated dental root canals

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Abstract

The high prevalence of *Enterococcus faecalis* in root canal treated teeth with post-treatment disease, as evidenced by both molecular and traditional culturing methods, suggests that this species may be a key player in endodontic treatment failure. This study aimed to detect virulence factors by phenotypic and western blotting tests, and virulence genes by PCR from 20 clinical strains of *E. faecalis* isolated from treated root canals of teeth with (10) or without (10) apical periodontitis. Moreover, genomic diversity of these strains was assessed by pulsed-field gel electrophoresis (PFGE) and rep-PCR. All 20 strains presented the *gel*E gene (gelatinase), but 10 of them did not hydrolyze gelatin. Seven of the 10 gelatinase-producing isolates were recovered from root canals with lesions, which suggests a role for this virulence factor in the pathogenesis of post-treatment disease. The *esp* gene was expressed only in cases where gelatinase production was negative. The other virulence genes were found in 90% (*efa*A and *ace* genes), 45% (*agg* gene) and 95% (*cpd* gene) of the *E. faecalis* isolates. As for PFGE and rep-PCR, no specific clonal type of *E. faecalis* was found in association with teeth with or without disease, revealing the interindividual clonal diversity of endodontic infections.

Keywords: *Enterococcus faecalis*; Endodontic infection; Virulence factors; Genotype patterns

1. Introduction

Traditional methods of microbial identification in endodontic infections have evidenced the presence of *Enterococcus faecalis*, especially in association with persistent/secondary infections (Pinheiro et al., 2003; Sundqvist et al., 1998). The consequent assumption that this species plays a major role in endodontic treatment failure resulted in various in vitro and ex vivo studies testing protocols to eliminate *E. faecalis* from root canals (Gomes et al., 2002; Vivacqua-Gomes et al., 2005).

Although molecular studies have shown that it is not a predominant species in cases of endodontic retreatment (Rôças et al., 2004; Sakamoto et al., 2008), *E. faecalis* has been confirmed as the most commonly found microorganism in persistent/secondary infections (Rôças et al., 2004; Siqueira and Rôças, 2004). However, *E. faecalis* has also been identified in cases with no apical periodontitis lesions (Molander et al., 1998). This was confirmed by molecular studies and led to the hypothesis that it may not necessarily be the main pathogen associated with post-treatment disease (Kaufman et al., 2005; Zoletti et al., 2006). The reason why *E. faecalis* is found in cases with and without disease remains to be clarified, but it may be related to differences in virulence abilities among strains of the species.

Since *Enterococcus* is not as virulent as other Gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae* or *Streptococcus pyogenes*, the study of its pathogenicity is more difficult (Koch et al., 2004). Although

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no study has thus far evidenced the specific role of virulence factors of enterococci in human infections, several of these factors have been described and studied (Eaton and Gasson, 2001; Jett et al., 1994). Candidate virulence factors of *E. faecalis* include: cytolysin and proteolytic enzymes (gelatinase and serine protease), adhesins (aggregation substance, enterococci surface protein or Esp, collagen adhesion protein or Ace, antigen A or EfaA) and capsular and cellular wall polysaccharides (Jett et al., 1994).

Gelatinase is a hydrophobic metalloprotease with the capacity for cleaving insulin, casein, hemoglobin, collagen, gelatin and fibrin (Waters et al., 2003). Studies have been performed in an attempt to associate its proteolytic properties to a higher occurrence of enterococci in endocarditis and bacteremia (Baldassari et al., 2004), urinary infections (Nakayama et al., 2002) and oral infections (Sedgley et al., 2004). However, the microorganism may not express gelatinase even in the presence of the *gel*E gene due to a chromosomal deletion of 23.9 kilobases in the locus *fsr* region (Roberts et al., 2004).

The enterococci surface protein (Esp) is encoded by the *esp* gene and may be involved in colonization and persistence of *E. faecalis* during infections (Koch et al., 2004; Shankar et al., 2001). It is likely that it mediates the primary interaction of the pathogen with host surfaces during biofilm formation (Tendolkar et al., 2004).

Since *E. faecalis* is highly prevalent in root canal treated teeth with apical periodontitis (Zoletti et al., 2006), it seems relevant to analyze possible involvement of virulence genes and factors, including gelatinase and enterococci surface protein, in the occurrence of this species in previously treated root canals associated or not with post-treatment disease, as well as to verify the biofilm formation ability of endodontic isolates. The observation of clonal differences among these isolates may also shed light on their participation in disease causation.

2. Materials and methods

2.1. Case description and sample-taking

Twenty strains of *E. faecalis* isolated from root canal treated teeth of 18 adult patients (ages ranging from 19 to 75 years) were used in this study. Among the isolates, 10 were from teeth that had no radiographic evidence of apical periodontitis and were referred to endodontic retreatment due to long exposure of root canal filling material to the oral cavity due to loss of coronal restoration, or when extensive coronal restoration had to be replaced and the technical quality of the endodontic treatment was considered inadequate. The other 10 isolates were from teeth with post-treatment apical periodontitis as revealed by radiographs. Clinical samples were collected from 85 patients who had been referred to the endodontic clinics of two universities (Federal University of Rio de Janeiro and Estácio de Sá University) for root canal retreatment. All root canal treated teeth had had endodontic therapy completed more than 1 year previously, and termini of the root canal filling ranging from 0 to 5 mm short of the radiographic root apex. Selected teeth showed an absence of periodontal pockets deeper than 4 mm.

All root canal samples were collected by one of the authors (G.O.Z.) after supragingival plaque removal by scaling, cleaning with pumice and isolation with a rubber dam of each tooth. Sample-taking was as described previously by Zoletti et al. (2006).

2.2. Culture and identification procedures

After incubation for 72 h at 35 °C, samples that showed growth in Enterococcosel broth (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) were plated onto blood agar plates (Plast Labor, Rio de Janeiro, RJ, Brazil) and incubated at 35 °C for 72 h. Pure colonies were subjected to conventional tests for identification of enterococcal species (Teixeira and Facklam, 2003). Identification was confirmed by PCR using *E. faecalis*-specific primers (see below).

2.3. Production of gelatinase

Production of gelatinase was determined on Todd-Hewitt agar (Difco, Laboratories, Le Pont de Claix, France) containing 30 g/L of gelatin, according to Coque et al. (1995). Colonies were spotted onto plates after overnight growth at 35 °C and placed at 4 °C for 5 h. Appearance of zones of turbidity around the colonies was indicative of hydrolysis. *E. faecalis* OG1RF was used as positive control.

2.4. SDS-PAGE and western blot analysis

SDS–7.5% polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970). After electrophoresis, gels were stained with Coomassie blue, destained, dried and transferred to nitrocellulose membranes (Hybond-C extra Amersham, GE Healthcare, Piscataway, NJ, USA) (Towbin et al., 1979). Membranes were incubated with anti-Esp rabbit immune serum (kindly provided by Dr. Nathan Shankar, University of Oklahoma, USA) diluted 1:250. Bound antibodies were detected as described by Jaffe et al. (1984) with DAB (3,3′ diaminobenzidine tetrahydrochloride dihydrate, 97%) (Sigma Chemical Co., St Louis, MO, USA).

2.5. Quantification of bacterial biofilm on a polystyrene surface (microtiter-plate adherence, or MPA)

The MPA assay was performed according to Stepanovic et al. (2007), with modifications. A 0.5 McFarland standard bacterial suspension was diluted 1:100 (v/v) in Tryptic Soy Broth–TSB (Becton) supplemented with 1% glucose (Merck, New Jersey, USA). Subsequently, 200 µL aliquots were distributed in wells of a microtiter plate of 96 wells (model 92096 TPP–"Techno Plastic Products", Trasadingen, Switzerland), and incubated for 24 h at 35 °C. Then, the content of each well was aspirated, and the well was washed four times with 100 µL of phosphate-buffered saline (pH 7.2). The adherent bacteria were stained for 1 min with 150 µL of

0.1% violet crystal solution. Excess stain was rinsed off by placing the plate under running tap water. *Staphylococcus epidermidis* strains ATCC 35984 (biofilm producer) and ATCC 12228 (non-biofilm producer) were used as controls. After the plates were overturned and air-dried, the dye bound to the adherent cells was resolubilized with 150 μL of 95% ethanol. The results were obtained using an ELISA reader model 680 (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 570 nm. Comparative analyses were performed according to Stepanovic et al. (2007) using the wells containing *S. epidermidis* ATCC 12228 (non-biofilm producer) as negative controls.

2.6. PCR procedures

One-milliliter aliquots from positive cultures in selective Enterococcosel broth (Becton) were transferred to microtubes and DNA was extracted by boiling (Siqueira and Rôças, 2004). Genes that encode for 16S rRNA of *E. faecalis*, aggregation substance (*agg*), enterococci surface protein (*esp*), gelatinase (*gel*E), gelatinase-negative phenotype determinant (*ef1841*/*fsr*C), endocarditis antigen (*efa*A), sex pheromones (*cpd*) and collagen binding antigen (*ace*) were targeted. Primer sequences and PCR conditions used for identification of *E. faecalis* and its virulence genes are shown in Table 1.

Prevalence of *E. faecalis* was recorded as the percentage of cases examined. The Fisher Exact Test was performed to analyze the association between enterococci virulence factors and periradicular disease. Significance was established at 5% ($p < 0.05$).

2.7. Genotyping by rep-PCR

The repetitive-sequence PCR (rep-PCR) was performed as follows. Endodontic isolates of *E. faecalis* were grown overnight at 35 °C, in 5 mL of brain-heart Infusion broth (Difco). The bacterial suspension was diluted 1:10 in sterile water and adjusted to an optical density of 600 nm. Afterwards, it was transferred to microtubes and the DNA was obtained as described by Pacheco et al. (1997). Aliquots of 1 μL of the DNA extracts were used as a template for PCR amplification with the primer RW3A as described by Namdari and DelVechio (1998) (Table 1).

2.8. Genotyping by PFGE

E. faecalis isolates were analyzed for clonal diversity by pulsed-field gel electrophoresis (PFGE) after *Sma*I (New England Biolabs, Inc., Beverly, MA, USA) digestion as described by Mondino et al. (2003) and Teixeira et al. (1997). Differences between isolates were determined by visual inspection of the bands, as recommended by Van Belkum et al. (2007) and by the Molecular Analyst Fingerprinting Plus software package (version 1.12) of the Image Analysis System (Bio-Rad, Hercules, CA, USA), using the Dice index and the unweighted pair group method with arithmetic averages for estimation of similarity and clustering.

3. Results

3.1. Isolation of E. faecalis

Of the 85 teeth included in the present study, four cases with lesions and one without lesions were excluded from the study because of contamination of the tooth crown by *Enterococcus* species as revealed by sterility controls. Results showed that, of the 80 teeth analyzed by culture, *E. faecalis* was detected in 20 (25%): 10 from treated root canals with apical periodontitis and the other 10 from treated teeth without disease.

3.2. Detection of virulence genes

Virulence genes possibly related to the persistence of *E. faecalis* in endodontic infections were analyzed in the 20 *E. faecalis* strains isolated from root canals with or without apical periodontitis (Table 2). The *gel*E gene was found in all

Table 1
Oligonucleotides used in this study for identification of *Enterococcus faecalis* and detection of different virulence genes by PCR.

Target DNA	Sequence of primers	Temperature conditions	Amplicon size (pb)	Reference
16S rDNA *E. faecalis*	5′-GTTTATGCCGCATGGCATAAGAG-3′ 5′-CCGTCAGGGGACGTTCAG-3′	95 °C–2 min; 36 cycles (95 °C–30 s; 60 °C–60 s; 72 °C–60 s) and 72 °C–2 min	310	Siqueira and Rôças, 2004
*gel*E	5′-ACCCCGTATCATTGGTTT-3′ 5′-ACGCATTGCTTTTCCATC-3′	94 °C–2 min; 35 cycles (92 °C–30 s; 52 °C–30 s; 72 °C–60 s) and 72 °C–2 min	419	Eaton and Gasson, 2001
esp	5′-TTGCTAATGCTAGTCCACGACCC-3′ 5′-GCGTCAACACTTGCATTGCCGAA-3′	94 °C–2 min; 35 cycles (92 °C–30 s; 52 °C–30 s; 72 °C–60 s) and 72 °C–2 min	933	Eaton and Gasson, 2001
agg	5′-AAGAAAAAGAAGTAGACCAAC-3′ 5′-AAACGGCAAGACAAGTAAATA-3′	94 °C–2 min; 35 cycles (92 °C–30 s; 56 °C–30 s; 72 °C–60 s) and 72 °C–2 min	1553	Eaton and Gasson, 2001
cpd	5′-TGGTGGGTTATTTTTCAATTC-3′ 5′-TACGGCTCTGGCTTACTA-3′	94 °C–2 min; 35 cycles (92 °C–30 s; 56 °C–30 s; 72 °C–60 s) and 72 °C–2 min	782	Eaton and Gasson, 2001
ace	5′-AAAGTAGAATTAGATCACAC-3′ 5′-TCTATCACATTCGGTTGCG-3′	94 °C–2 min; 35 cycles (92 °C–30 s; 56 °C–30 s; 72 °C–60 s) and 72 °C–2 min	320	Duprè et al., 2003
*efa*A	5′-CTGGAGAAAGAAATGGAGGA-3′ 5′-CTACTAACACGTCACCAATG-3′	94 °C–2 min; 35 cycles (92 °C–30 s; 56 °C–30 s; 72 °C–60 s) and 72 °C–2 min	499	Duprè et al., 2003
ef1841/fsrC	5′-GATCAAGAAGGGAAGCCACC-3′ 5′-CCAACCGTGCTCTTCTGGA-3′	94 °C–2 min; 35 cycles (92 °C–30 s; 56 °C–60 s; 72 °C–2 min) and 72 °C–5 min	1050	Nakayama et al., 2002

Table 2
Clinical characteristics and virulence and genotypic patterns associated with 20 *E. faecalis* isolates from treated root canals.

Case	Sex	Age (y)	Tooth	Lesion	Lesion diameter (mm)	Apical limit of the root filling (mm short)	Length of treatment (y)	Virulence patterns										PFGE patterns	rep-PCR patterns
								Gelatinase			Surface protein		Other genes[a]				Biofilm formation[c]		
								*gel*E gene[a]	gelatin hydrolysis[b]	ef1841/*fsr*C gene[a]	*esp* gene[a]	gene expression[d]	*efa*A	*ace*	*agg*	*cpd*			
RW12	F	28	25	Yes	1	1	5	+	−	+	+	+	+	+	−	−	++	C1	b
RW35*	M	38	12	Yes	2	0	6	+	+	−	−	−	+	+	−	+	+++	A	a
RW37*	M	38	22	Yes	5	3	4	+	+	−	−	−	+	+	+	+	++	A	a
RW68	M	28	22	Yes	1	1	>10	+	+	−	−	−	+	+	−	+	++	F	a
RW72	F	41	25	Yes	3	0	10	+	−	+	+	+	+	+	+	+	+++	G	a
RW77	F	28	36D	Yes	3	0	8	+	−	+	+	+	+	+	+	+	+	D1	b
RW79	F	23	22	Yes	2	0	3	+	+	−	−	−	+	+	+	+	+	H	a
RW80	F	27	46D	Yes	1	4	2	+	+	−	−	−	+	+	−	+	+++	I	b
RW81	M	43	46M	Yes	2	1	2	+	+	−	−	−	+	+	−	+	+	J	a
RW85	M	33	24V	Yes	2	3	3	+	+	−	−	−	+	+	+	+	++	K	a
RN04	F	43	11	No	−	1	5	+	−	+	−	−	+	+	−	+	++	L	c
RN18	M	45	22	No	−	4	>5	+	+	−	−	−	+	+	−	+	++	M	d
RN29	M	26	15	No	−	1	2	+	+	−	+	−	+	+	−	+	+++	N	d
RN44	F	39	11	No	−	0	>6	+	−	+	+	+	+	+	−	+	+	D2	f
RN45	F	45	25	No	−	1	>5	+	−	+	−	−	+	+	+	+	+++	E1	c
RN50**	M	29	22	No	−	3	>20	+	−	+	+	+	−	−	−	+	+	B	e
RN55**	M	29	21	No	−	2	>5	+	−	+	+	+	−	−	−	+	+	B	e
RN66	F	44	27P	No	−	0	5	+	−	+	−	−	+	+	+	+	++	E2	g
RN73	M	26	46D	No	−	1	6	+	−	+	+	+	+	+	+	+	+	C2	h
RN74	M	28	15	No	−	0	3	+	+	−	−	−	+	+	+	+	+++	O	h

(F) female; (M) male; (y) in years. *gel*E gene−encode a gelatinase; *esp* gene−encode an enterococci surface protein; *fsr*/ef1841−encode a gelatinase-negative phenotype determinant; *efa*A−encode a antigen A; *ace*− encode a collagen adhesion protein; *agg*−encode an aggregation substance; *cpd*−encode a sex pheromones; (*) (**) strains of *E. faecalis* isolated from the different root canals treated in a same patient.

[a] Detected by PCR (polymerase chain reaction).

[b] Phenotypic test performed in Todd-Hewitt agar with 30 g/L of gelatin.

[c] Detected by microtiter-plate adherence.

[d] Detected by western blotting

isolates; the *cpd* gene was detected in 19 (95%); *efa*A and ace genes were found in 18 (90%); the *agg* gene was found in 9 (45%); and the *esp* gene was found in 8 (40%) out of the 20 isolates analyzed.

3.3. Phenotypic characterization of gelatinase production

A difference between carriage of the gelatinase gene and its expression was noticed. Although all isolates had this gene, only half of them exhibited an opaque halo indicative of positiveness for the gelatinase phenotypic test (Table 2). Of the positive cases, seven (70%) were from root canal treated teeth with lesions and only three (30%) were from cases without lesions. Gelatinase expression was confirmed by the absence of detection of the *ef1841/fsrC* gene in the positive isolates.

3.4. Expression of the Esp virulence factor

Western blotting using anti-Esp polyclonal rabbit serum showed that 7 of 8 (87.5%) isolates in which the *esp* gene was identified reacted with the antibody, indicating production of this adhesin by the isolates (Table 2). All strains positive for *esp* gene expression were negative for gelatinase production, except for 3 strains from teeth without lesions, which expressed neither.

3.5. Biofilm formation

All strains of *E. faecalis* isolated from treated root canals were biofilm producers. There was no correlation between the intensity of biofilm production by strains detected in the presence or absence of disease. In cases with apical periodontitis, *E. faecalis* strains were classified as follows: 3 weak, 4 moderate and 3 strong biofilm producers. In cases without disease, strains were classified as follows: 4 weak, 3 moderate and 3 strong biofilm producers.

3.6. Rep-PCR

Genetic polymorphism was performed using rep-PCR with the clinical strains isolated from root canal treated teeth and the control strains *E. faecalis* DS16, *E. faecalis* V583 and *E. faecalis* OG1RF. Banding profiles for these strains showed 6–9 bands and revealed wide clonal diversity. The 20 strains were distributed into 8 different clonal groups: genotypes a and b corresponded to 7 and 3 isolates, respectively; genotypes c, d, e and h were represented by 2 isolates each; and genotypes f an g comprised one isolate each. The isolates included in the same genotype had an identical profile, and were classified into another genotype according to the identification of at least one different band.

3.7. PFGE

PFGE analysis of genomic DNA from the 20 *E. faecalis* endodontic isolates revealed 18 restriction profiles clustered into 14 different genotypes, with 50% of the isolates included in five genotypes (Fig. 1). Genotype A included isolates 35RW and 37RW, whereas genotype B comprised isolates 50RN and 55RN; both strains of each genotype were isolated from the same patient and were considered as indistinguishable. Isolates 45RN (subtypes C1) and 66RN (subtype C2) differed by four bands; 77RW (subtype D1) and 44RN (subtype D2) also differed by four bands; 12RW (subtype E1) and 73RN (subtype E2) differed by two bands. The remaining strains were considered unrelated because they differed by more than 5 bands, and were classified into genotypes F to O.

4. Discussion

A higher prevalence of *E. faecalis* has been observed in both primary and persistent endodontic infections through the use of molecular methods (Rôças et al., 2004; Sassone et al., 2007). However, the role of *E. faecalis* in the pathogenicity of apical periodontitis is still uncertain. Studies evaluating its prevalence in root canal treated teeth with or without post-treatment disease concluded that, although *E. faecalis* has been associated with treated root canals, a causal relationship with post-treatment disease remains undetermined (Kaufman et al., 2005; Zoletti et al., 2006).

In addition to the characteristics of resistance and the capacity to survive endodontic treatment (Love, 2001; Sedgley et al., 2005), *E. faecalis* presents virulence traits that, in sufficient concentrations, may enable the microorganism to remain in the root canal and either directly or indirectly inflict damage to the periradicular tissues. Among such virulence factors, gelatinase and enterococci surface protein (Esp) have greater potential for participating in colonization and disease causation (Kristich et al., 2004; Sedgley et al., 2004).

In the present study, all 20 isolates of *E. faecalis* recovered from treated root canals carried the *gel*E gene, but only 10 of them (50%) hydrolyzed gelatin in the phenotypic test. It is important to emphasize that gelatinase expression occurred in 70% (7/10) of the strains isolated from teeth with disease, compared to only 30% (3/10) of isolates from teeth without lesions, although it is not statistically significant ($p = 0.19$). This suggests a role for this virulence factor in the pathogenesis of post-treatment apical periodontitis.

Moreover, it is possible to infer a relationship between gelatinase production and biofilm-forming ability and with the perpetration of the infectious process and bacterial persistence in the root canal, since 5 of the 7 gelatinase-producing strains found in disease cases were moderate to strong biofilm producers. Although the role of gelatinase in enhancing biofilm formation is still unknown, Kristich et al. (2004) described possible models: gelatinase might participate in production of an extracellular signaling peptide by proteolytically processing an inactive secreted peptide precursor to a mature component, or it might proteolytically activate another surface protein involved in some aspects of regulation or the process of biofilm development, such as a protein that participates in secretion of extracellular polymeric matrix material.

Expression of gelatinase is regulated by a quorum sensing system encoded by a chromosomal deletion of 23.9 kilobases

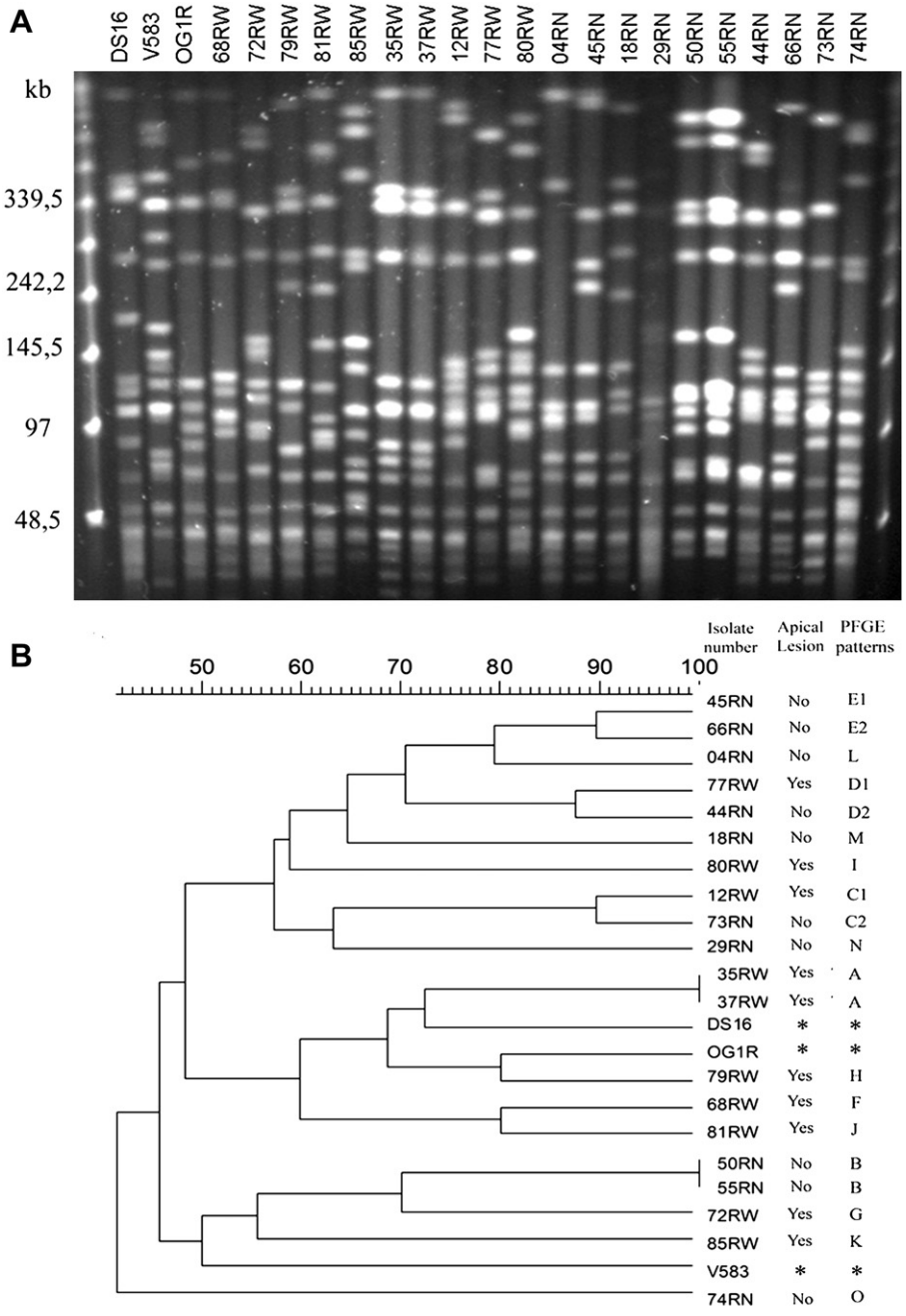

Fig. 1. (A) PFGE genotypes of 20 strains of *Enterococcus faecalis* isolated from root canal treated with and without post-treatment disease. (B) Dendogram resulting from the computer-assisted analysis on the PFGE profiles. *Control strains.

in the locus *fsr* region (Roberts et al., 2004). This was also supported by our results, since the *ef1841/fsr* gene was detected only in strains with negative phenotypes for gelatinase production.

The *esp* gene that encodes a surface protein was found in 8 (40%) of the strains analyzed, 5 from teeth without lesions and 3 from teeth with disease ($p = 0.67$). Expression of this enterococcal surface protein was confirmed by western blotting and results showed that only one of the eight *esp* gene-positive isolates did not express this factor. Interestingly, the Esp factor was expressed only by strains that did not produce gelatinase. Three isolates expressed neither of these virulence factors. However, these strains were from cases without lesion, and two of them belonged to the same PFGE genotype (strains RN45 and RN66). Although the gene *esp* occurred similarly in cases without (40%) and with (30%) post-treatment apical periodontitis, a relationship between this virulence factor and the presence of *E. faecalis* in the root canal may exist. The Esp factor could act as an alternative tool to gelatinase, as for root canal colonization by *E. faecalis*.

Recent studies have shown that the presence of Esp is not a determinant in formation of biofilm, even when associated with other virulence factors (Carniol and Gilmore, 2004; Kristich et al., 2004). Our results obtained for analysis of

biofilm production by endodontic strains of *E. faecalis* reinforce this observation, since 5 of the 7 strains that expressed Esp were classified as weak biofilm producers.

The analysis of polymorphism through rep-PCR enabled the distribution of strains into eight different genotypes. Two bands of about 1200 and 1300 bp were found in all strains of *E. faecalis* analyzed (Namdari and DelVechio, 1998). According to rep-PCR analysis, 10 strains recovered from cases with disease were included in two major groups (genotypes a and b). However, rep-PCR analysis showed lower discriminatory power when compared to PFGE. Actually, some of the clinical strains presenting the same genotype by rep-PCR showed distinct genotypic profiles by PFGE (Fig. 1). These differences may be related to the low number of bands generated and the poor reproducibility of the rep-PCR method, as already reported by Malathum et al. (1998).

PFGE analysis of the 20 *E. faecalis* strains resulted in 18 restriction profiles clustered into 14 different genotypes. About 50% of the strains were included in five genotypes (A–E). In spite of the large clonal diversity found among *E. faecalis*, four isolates from two genotypes (A and B) were classified as similar by both rep-PCR and PFGE methods. In fact, isolates 35RW and 37RW were from different teeth, but the same patient. A similar finding was obtained for isolates 50RN and 55RN. Three other genotypes presented closely related PFGE subtypes. These relationships occurred between isolates from genotypes C and D from root canals with or without apical periodontitis, and from genotype E, comprising isolates from cases without disease. Furthermore, except for strains from genotype E, the other strains classified within clonal groups showed distinct virulence and biofilm patterns, although they presented similar profiles for the *gel*E and *esp* gene expression, demonstrating the clonal variability of *E. faecalis* strains isolated from treated root canals.

In conclusion, the present study provides information on the genetic and phenotypic aspects of virulence traits of clinical strains of *E. faecalis* isolated from root canal treated teeth with or without apical periodontitis. No prevalent genotype was identified, which was a result of the high interindividual variability observed. Some of the virulence characteristics detected in this study may help to explain the involvement of *E. faecalis* in the establishment of post-treatment disease, probably as part of a mixed microbial community presenting complex interactions that may influence disease progression and outcome.

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References

Baldassari, L., Creti, R., Arciola, C.R., Montanaro, L., Venditti, M., Di Rosa, R., 2004. Analysis of virulence factors in cases of enterococcal endocarditis. Clin. Microbiol. Infect. 10, 1006–1008.

Carniol, K., Gilmore, M., 2004. Signal transduction, quorum-sensing, and extracellular protease activity in *Enterococcus faecalis* biofilm formation. J. Bacteriol. 186, 8161–8163.

Coque, T.M., Patterson, J.E., Stecklberg, J.M., Murray, B.E., 1995. Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J. Infect. Dis. 171, 1223–1229.

Duprè, I., Zanetti, A.M., Schito, G., Fadda, G., Sechi, L.A., 2003. Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). J. Med. Microbiol. 52, 491–498.

Eaton, T.J., Gasson, M.J., 2001. Molecular screening of *Entercoccus* virulence determinants and potential for genetic exchanges between food and medical isolates. Appl. Environ. Microbiol. 67, 1628–1635.

Gomes, B.P.F.A., Ferraz, C.C.R., Garrido, F.D., Rosalen, P.L., Zaia, A.A., Teixeira, F.B., Souza-Filho, F.J., 2002. Microbial susceptibility to calcium hidroxide pastes and their vehicles. J. Endod. 28, 758–761.

Jaffe, C.L., Bennett, E., Grimaldi, J.R., Mcmahon-Pratt, D., 1984. Production and characterization of species-specific monoclonal antibodies against *Leishmania donovani* for immunodiagnostics. J. Immunol. 133, 440–447.

Jett, B.D., Huycke, M.M., Gilmore, M.S., 1994. Virulence of enterococci. Clin. Microbiol. Rev. 7, 462–478.

Kaufman, B., Spangberg, L., Barry, J., Fouad, A.F., 2005. *Enterococcus* spp. in endodontically treated teeth with and without periradicular lesions. J. Endod. 31, 851–856.

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

Koch, S., Hunfnagel, M., Theilacker, C., Huebner, J., 2004. Enterococcal infections: host response, therapeutic and prophylatic possibilities. Vaccine 22, 822–830.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 333, 330–334.

Love, R.M., 2001. *Enterococcus faecalis*—a mechanism for its role in endodontic failure. Int. Endod. J. 34, 399–405.

Malathum, K., Singh, K.V., Weinstock, G.M., Murray, B.E., 1998. Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. J. Clin. Microbiol. 36, 211–215.

Molander, A., Reit, C., Dahlén, G., Kvist, T., 1998. Microbiological status of root-filled teeth with apical periodontitis. Int. Endod. J. 31, 1–7.

Mondino, S.S.B., Castro, A.C.D., Mondino, P.J., Carvalho, M.G., Silva, K.M., Teixeira, L.M., 2003. Phenotypic and genotypic characterization of clinical and intestinal enterococci isolated from inpatients and outpatients in two brazilian hospitals. Microbiol. Drug Resist. 9, 167–174.

Nakayama, J., Kariyama, R., Kumon, H., 2002. Description of a 23.9-kilobase chromosomal deletion containing a region encoding *fsr* genes which mainly determines the gelatinase-negative phenotype of clinical isolates of *Enterococcus faecalis* in urine. Appl. Environ. Microbiol. 68, 3152–3155.

Namdari, H., DelVechio, V., 1998. Application of PCR for characterization of Enterococci. Clin. Microbiol. Newsletter 20, 91–98.

Pacheco, A.B.F., Guth, B.E., Soares, K.C.C., Nishimura, L., Almeida, D.F., Ferreira, L.C.S., 1997. Random amplification of polymorphic DNA reveals serotype-specific clonal clusters among enterotoxigenic *Escherichia coli* strains isolated from humans. J. Clin. Microbiol. 35, 1521–1525.

Pinheiro, E.T., Gomes, B.P.F.A., Ferraz, C.C.R., Teixeira, F.B., Zaia, A.A., Souza-Filho, F.J., 2003. Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility. Oral Microbiol. Immunol. 18, 100–103.

Roberts, J.C., Singh, K.V., Okhuysen, P.C., Murray, B.E., 2004. Molecular epidemiology of the *fsr* lócus and of gelatinase production among different subsets of *Enterococcus faecalis* isolates. J. Clin. Microbiol. 42, 2317–2320.

Rôças, I.N., Siqueira Jr., J.F., Santos, K.R.N., 2004. Association of *Enterococcus faecalis* with different forms of periradicular diseases. J. Endod. 30, 315–320.

Sakamoto, M., Siqueira Jr., J.F., Rôças, I.N., Benno, Y., 2008. Molecular analysis of the root canal microbiota associated with endodontic treatment failures. Oral Microbiol. Immunol. 23, 275–281.

Sassone, L., Fidel, R., Figueiredo, L., Fidel, S., Faveri, M., Feres, M., 2007. Evaluation of the microbiota of primary endodontic infections using checkerboard DNA–DNA hybridization. Oral Microbiol. Immunol. 22, 390–397.

Sedgley, C.M., Lennan, S.L., Clewell, D.B., 2004. Prevalence, phenotype and genotype of oral enterococci. Oral Microbiol. Immunol. 19, 95–101.

Sedgley, C.M., Lennan, S.L., Appelbe, O.K., 2005. Survival of *Enterococcus faecalis* in root canals *ex vivo*. Int. Endod. J. 38, 735–742.

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

Siqueira Jr., J.F., Rôças, I.N., 2004. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 97, 85–94.

Stepanovic, S., Vulkovic, D., Hola, V., Di Bonaventura, G., Djukic, S., Cirkovic, I., Ruzicka, F., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 115, 891–899.

Sundqvist, G., Figdor, D., Persson, S., Sjogren, U., 1998. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative retreatment. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 85, 86–93.

Tendolkar, P.M., Baghdayan, A.S., Gilmore, M.S., Shankar, N., 2004. Enterococcal surface protein, esp, enhances biofilm formation by *Enterococcus faecalis*. Infect. Immunol. 72, 6032–6039.

Teixeira, L.M., Carvalho, M.G.S., Merquior, V.L.C., Steigerwalt, Teixeira, M.G.M., Brenner, D.J., Facklam, R.R., 1997. Recent approaches on the taxonomy of the enterococci and some related microorganisms. Adv. Exp. Med. Biol. 418, 387–391.

Teixeira, L.M., Facklam, R.R., 2003. Enterococcus. In: Murray, P.R., Baron, E. J., Jorgensen, J.H., Pfaller, M.A., Yolken, R.H. (Eds.), Manual of Clinical Microbiology, eighth ed. ASM Press, Washington, DC, pp. 422–433.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.

Van Belkum, A., Tassios, P.T., Dijkshoom, L., Haeggman, S., Cookson, B., Fry, N.K., Fussing, V., Green, J., Feil, E., Gemer-Smidt, P., Brisse, S., Struelens, M., 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin. Microbiol. Infect. Diseases 13, 1–46.

Vivacqua-Gomes, N., Gurgel-Filho, E.D., Gomes, B.P.F.A., Ferraz, C.C.R., Zaia, A.A., Souza-Filho, F.J., 2005. Recovery of *Enterococcus faecalis* after single-or multiple-visit root canal treatments carried out in infected teeth *ex vivo*. Int. Endod. J. 38, 697–704.

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

Zoletti, G.O., Siqueira Jr., J.F., Santos, K.R.N., 2006. Identification of *Enterococcus faecalis* in root-filled teeth with or without periradicular lesions by culture dependent and—independent approaches. J. Endod. 32, 722–726.