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Investigation *in vivo* of *Enterococcus faecalis* in endodontic retreatment by phenotypic and genotypic methods

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ABSTRACT. This study aimed to investigate the prevalence of *E. faecalis* in root-filled canals using culture and molecular approaches. It was evaluated the antimicrobial susceptibility to different antibiotics and the virulence factors of *E. faecalis* isolates. Microbial samples were taken from thirty root-filled canals. Culture methods and 16S rDNA assay were used to identify *E. faecalis*. The antimicrobial susceptibility of *E. faecalis* was determined by MIC values using the E test. Cultivable strains of *E. faecalis* were investigated for virulence factors by PCR technique. *E. faecalis* were detected by culture (7/30), traditional PCR assay (13/30) and nested PCR (23/30). Both PCR were significantly more effective than culture in detecting *E. faecalis* ($p < 0.05$). All tested *E. faecalis* were highly sensitive to amoxicillin. Some strains of *E. faecalis* were resistant to antibiotics such as rifampicin (4/12), erythromycin (3/12) and azythromycin (8/12). The genes *efaA* and *ace* were detected in all isolates. The other virulence genes were found in 91.6 (*gelE*), 83.3 (*asa*), 25 (*esp*) and 16.6% (*cylA*). Strains of *E. faecalis* isolated from root-filled canals showed virulence factors related to adherence. They also showed resistance to some antibiotics commonly used in dentistry.

Keywords: endodontics, antibiotics, antimicrobial susceptibility, virulence.

Investigação *in vivo* de *Enterococcus faecalis* em retratamento endodôntico por meio de métodos fenotípicos e genotípicos

RESUMO. O objetivo deste estudo foi investigar a prevalência de *E. faecalis* em dentes tratados endodonticamente utilizando as técnicas de cultura e molecular. Foram avaliados a suscetibilidade antimicrobiana frente a diferentes antibióticos e os fatores de virulência de *E. faecalis* isolados. As amostras foram coletadas de 30 dentes tratados endodonticamente. Métodos de cultura e 16S rDNA foram utilizados para identificar *E. faecalis*. A suscetibilidade antimicrobiana de *E. faecalis* foi determinada por valores de MIC, utilizando o E test. Os fatores de virulência de cepas de *E. faecalis* cultiváveis foram investigados pela técnica de PCR. *E. faecalis* foram detectados por cultura (7/30), PCR tradicional (13/30) e nested PCR (23/30). Ambas as técnicas de PCR foram significativamente mais eficazes do que a cultura na detecção de *E. faecalis* ($p < 0,05$). Todos os *E. faecalis* testados foram altamente sensíveis à amoxicilina. Algumas cepas de *E. faecalis* foram resistentes a antibióticos, como a rifampicina (4/12), eritromicina (3/12) e azitromicina (8/12). Os fatores de virulência *efaA* e *ace* foram detectados em todos os isolados. Os outros genes de virulência foram encontrados em 91,6 (*gelE*), 83,3 (*asa*), 25 (*esp*) e 16,6% (*cylA*). As cepas de *E. faecalis* isoladas em dentes tratados endodonticamente mostraram fatores de virulência relacionados à adesão. Eles também apresentaram resistência a alguns antibióticos comumente utilizados na odontologia.

Palavras-chave: endodontia, antibióticos, suscetibilidade antimicrobiana, virulência.

Introduction

The bacteria remaining in the root canal system after endodontic treatment cause secondary, or persistent, infections (BYSTRÖM et al., 1987). These microorganisms may have survived the biomechanical procedures or invaded the canal via coronal leakage of the root filling. Bacterial cultures and molecular studies have confirmed that *Enterococcus faecalis* is one of the most prevalent bacteria found in the root canal after endodontic treatment (PINHEIRO et al., 2003a; STUART et al., 2006; SCHIRRMEISTER et al., 2007).

Attention has been given to enterococci since 1970's when they were recognized as major nosocomial pathogens causing bacteremia, endocarditis, bacterial meningitis and various other

infections (STUART et al., 2006). There are also increasing evidences of the appearance of multi-antibiotic resistant strains. They have also been associated with endodontic infections and are frequently isolated from root-filled canals of teeth with chronic apical periodontitis (PINHEIRO et al., 2003a). *E. faecalis*, intrinsically or via acquisition, may be resistant to a wide range of antibiotics (SHEPARD; GILMORE, 2002), which, if used, may alter the microbiota in favor of *E. faecalis*.

In Dentistry, antibiotics are not generally used to treat chronic infections, such as apical periodontitis, in root-filled teeth (PINHEIRO et al., 2004). Usually, they are applied when patients present with progressive, diffuse swelling and systemic signs of infection including fever, malaise, and lymphadenopathy. Systemic antibiotics may also be used as a prophylactic measure for medically compromised patients. Despite these treatment guidelines, dental practitioners tend to overprescribe antibiotics in their practice, often without sufficient grounds for choosing a particular drug (RODRIGUEZ-NÚÑEZ et al., 2009). The increasing resistance of bacteria to some widely used antibiotics ensures the need for monitoring susceptibility patterns periodically by using susceptibility tests. The Epsilometer test (E test), an agar diffusion susceptibility test, holds the promise of being accurate and flexible enough to be performed in most clinical laboratories (SANCHES; JONES, 1992). Thus, it is prudent to study changes in the antimicrobial susceptibilities of endodontic pathogens to facilitate the choice of an appropriate antibiotic when indicated for the treatment of infections.

Virulence factors confer the traits that provide survival advantages to organisms in unusual environments. The characteristics of resistance and the capacity to survive endodontic treatment (LOVE, 2001; SEDGLEY et al., 2004) may enable *E. faecalis* to remain in the root canal and either directly or indirectly inflict damage to the periradicular tissues.

The present study aimed to investigate the prevalence of *E. faecalis* in root-filled canals with periapical lesions using culture technique, traditional PCR and nested PCR. Moreover, it was evaluated the antimicrobial susceptibility to different antibiotics and virulence factors of *E. faecalis* isolates.

Methods

Patient selection

Thirty patients were selected from those who attended the Piracicaba Dental School, São Paulo State, Brazil, with a need for nonsurgical endodontic retreatment. The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved a protocol describing the specimen collection for this investigation, and all patients signed an informed consent to participate. A detailed medical and dental history was obtained from each patient. Patients who had received antibiotic treatment during the last 3 months or had a general disease were excluded from the study. The age of the patients ranged from 19 to 65 years. All selected teeth had been previously single root-filled and showed radiographic evidence of apical periodontitis. Failure of root canal treatment was determined on the basis of clinical and radiographic examinations. All teeth had been root canal treated filled more than 2 years ago and the patients presented with asymptomatic. All teeth had enough crown structure for adequate isolation with a rubber dam, and showed no periodontal pockets deeper than 4 mm.

Microbial sampling

The teeth were isolated with a rubber dam. The crown and the surrounding rubber dam were disinfected with 30% H_2O_2 (v v^{-1}) for 30 s followed by 2.5 NaOCl for an additional 30 s. Subsequently, 5% sodium thiosulphate was used to inactivate the disinfectant agents (PINHEIRO et al., 2003a; 2004; GOMES et al., 2004; 2008). A swab sample was taken from the surface and streaked on blood agar plates to test for disinfection. An access cavity was prepared with sterile high-speed diamond burs under irrigation with sterile physiological solution. Before entering the pulp chamber, the access cavity was disinfected with the same protocol as above and the sterility again checked by taking a swab sample of the cavity surface and streaking onto blood agar plates. Aseptic techniques were used throughout root canal treatment and sample acquisition. The initial samples were collected with three sterile paper points, which were consecutively placed into each canal to the full length of the root, kept in place for 60 s and then pooled in a sterile tube containing 1 mL VMGA III transport medium. The samples were transported to the microbiology laboratory within 15 min. to an anaerobic workstation (DonWhitley Scientific, Bradford, UK).

Clinical procedures

The same endodontic specialist performed all retreatments and sampling procedures. The tooth was anesthetized and after accessing the pulp chamber, root filling materials were removed using crown-down technique. No solvent was used at any

time to avoid a negative effect on microbial viability. Radiographs performed in bucco-lingual and mesio-distal directions for each tooth were taken to confirm gutta-percha removal.

Root-filling materials were removed by rotary instrumentation and K-files (Dentsply Maillefer, Ballaigues, Switzerland) in a crown-down technique without the use of chemical solvents. Irrigation with sterile saline solution was performed to remove any remaining treatment materials and to moisten the canal prior to sample collection. A K-file size #15 was used to negotiate the root canal. The working length (full length of the canal) was established radiographically and with the aid of an electronic apex locator (Novapex, Forum Technologies, Rishon le-Zion, Israel). Further close inspection under high magnification with the dental operating microscope (D F Vasconcellos S/A, São Paulo State, Brazil) showed the complete removal of gutta-percha. After this, microbial samples were taken with three paper points, which were pooled into the VMGA III transport medium.

Microbial culturing and identification

Microbial samples, isolation and speciation were done using advanced microbiological techniques for anaerobic species.

Inside the anaerobic workstation, the tubes containing the transport medium were shaken in a mixer for 60 s (Agitador MA 162-MARCONI, São Paulo, São Paulo State, Brazil). Serial 10-fold dilutions were made up to 1 10^{-4} in pre-reduced Fastidious Anaerobe Broth (FAB, Laboratory M, Bury, UK) and 50 μL of each serial dilution were plated onto several media, as follows: 5% defibrinated sheep blood-FAA Agar (FAA, Laboratory M, Bury, UK) alone, and supplemented with 600 μL of hemin and 600 μL of menadione. Plates were incubated at 37°C in an anaerobic atmosphere for up to 48 hours to allow anaerobic or facultative microorganisms growth. In addition, 50 μL of initial sample was plated onto m-Enterococcus agar (Difco, Maryland, USA) and Mitis salivarius agar (Difco, Maryland, USA) to increase the chance of finding *Enterococcus faecalis*.

Preliminary characterization of microbial species was based on the features of the colonies (i.e. size, color, shape, high, lip, surface, texture, consistency, brightness and hemolysis), visualized under a stereoscopic lens (Lambda Let 2, Atto instruments Co., Hong Kong) at 16x magnification. Isolates were then purified by subculture, gram-stained, tested for catalase production, and their gaseous requirements established by incubation for 2 days aerobically and anaerobically. Based on this information, it was possible to select appropriate procedures for identification of *E. faecalis* using API 20 Strep (BioMérieux SA, Marcy-l'Etoile, France) for streptococci (Gram-positive cocci, catalase-negative). The detection system API 20 Strep is based on fermentative and biochemical properties of facultative anaerobic microorganisms being identified by standardized enzymatic reactions.

Antimicrobial susceptibility test

The antimicrobial susceptibility of isolates was investigated by means of the E test System (AB Biodisk, Solna, Sweden).

The strains of *E. faecalis* isolated (n = 12), which are facultative anaerobic Gram-positive cocci, were tested for their susceptibility/resistance to 12 antibiotics: benzylpenicillin, amoxicillin, amoxicillin + clavulanic acid, erythromycin, azithromycin, vancomycin, chloramphenicol, tetracycline, doxycycline, moxifloxacin, ciprofloxacin and rifampicin. The E test consists of strips containing different concentrations of an antimicrobial agent which can be placed directly on the agar plate. This test was evaluated by using Mueller-Hinton agar plates (Oxoid, Basingstoke, UK) 4 mm thick. Inocula were prepared by suspending growth on plates in Fastidious Anaerobe Broth (Laboratory M, Bury, UK) to a McFarland turbidity of 0.5. Sterile cotton swabs were used to inoculate plates, to which E test strips were then applied within 20 min. of inoculation. The surface of the plate was swabbed in three directions to ensure a complete distribution of the inoculum over the entire plate. Plates were incubated in an aerobic incubator for aerobic bacteria (36°C for 24 hours). All the tests were completed in duplicate. The susceptibility result was interpreted by comparing the minimum inhibitory concentration (MICs) of isolates with MIC interpretive standards established by National Committee for Clinical Laboratory Standards (NCCLS). After 24 hours of incubation under aerobic conditions and 10% CO_2 the concentration of the drug that inhibits 90% of bacterial growth in-vitro (MIC) could be easily read from the strip (CITRON et al., 1991).

The E test is based on the diffusion of a continuous, exponential concentration gradient of the antimicrobial agent from a plastic strip containing the antibiotic. After incubation of the E test strip on agar media with a lawn of bacteria, an ellipse of inhibition is formed around the strip.

The MICs were read from the intercept at which the ellipse inhibition zone intersected with the scale. The MICs including 90 and 50% of the strains were calculated. The diameter of the inhibition zone of

each strain was measured and the strains were graded as sensitive (S), intermediate (I) and resistant (R) according to the guidelines of NCCLS (2008).

Bacterial Detection (Polymerase chain reaction - PCR 16S rDNA)

DNA extraction: Microbial DNA from all samples and control sample from *Enterococcus faecalis* (ATCC 4034) were extracted and purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration (absorbance at 260 nm) was determined with a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA).

PCR assay: *E. faecalis* was identified using PCR amplification of signature sequences of the 16S rDNA gene. The oligonucleotide species-specific primers for *Enterococcus faecalis* were 5'-CCG AGT GCT TGC ACT CAA TTG G-3' (forward primer) and 5'-CTC TTA TGC CAT GCG GCA TAA AC-3' (reverse primer), producing a PCR amplicon of 138 bp (SEDGLEY et al., 2005). The PCR reaction was performed in a thermocycler (GenePro, Bioer Technology, Hangzhou, China) at a total volume of 25 containing 2.5 μL of 10X *Taq* buffer (1X) (Invitrogen, Carlsbad, CA, USA), 0.5 μL of dNTP mix (25 μM of each deoxyribonucleoside triphosphate – dATP, dCTP, dGTP and dTTP) (Invitrogen, Carlsbad, CA, USA), 1.25 μL of 25 mm $MgCl_2$, 0.25 μL of forward and reverse universal primers (0.2 μM) (Invitrogen, Carlsbad, CA, USA), 1.5 μL sample DNA, 1.5 μL Taq DNA polymerase (1 unit) (Invitrogen, Carlsbad, CA, USA), and 17.25 μL nuclease-free water. The temperature profile for the universal reaction included an initial denaturation step at 95°C for 2 min. followed by 36 cycles of a denaturation step at 95°C for 1 min., a primer annealing step at 57°C for 1 min., an extension step at 72°C for 1 min., and a final step at 72°C for 7 min. PCR products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, and viewed under ultraviolet transilluminating light. Either positive or negative detection of *E. faecalis* strains was based on the presence of clear bands of expected molecular sizes.

Nested PCR: DNA isolated from the root canal specimens were first amplified with prokaryotic universal ribosomal 16 and 23 s primers (785 and 422 respectively), as described elsewhere (MCCLELLAN et al., 1996; KUMAR et al., 2003; GOMES et al., 2008). The sequence of primers 785 and 422 were GGA TTA GAT ACC CTG GTA GTC (universal primer/16S/785 bp from 5'end/Forward) and GGA GTA TTT AGC TT (universal primer/23S/422 bp from 5'end/Forward), respectively. This amplification included the 16S rDNA and the downstream intergenic spacer region (ISR). Inclusion of the ISR provided an additional check of the specificity of the primers, because the length of this region varies among species. PCR reactions were performed in a total volume of 50 μL containing 1.25 U *Taq* DNA polymerase (Perkin-Elmer, Foster City, CA, USA), 5 μL of 10X PCR buffer plus 3 mm $MgCl_2$, 0.25 mm of each primer and 0.2 mm (each) deoxynucleoside triphosphates. For each sample, 0.5 μL of extracted DNA was added to the reaction mixture. PCR was also performed using a positive control (0.5 μL of DNA extracted from the *E. faecalis* strain ATCC 4034) and a negative control (only the reaction mixture without DNA). Samples were subjected to 22 cycles of denaturation at 94°C for 1 min., annealing at 42°C for 2 min., and primer extension at 72°C for 3 min., and a final extension of 72°C for 10 min., in a thermocycler (GenePro, Bioer Technology, Hangzhou, China). *E. faecalis* was then identified by a second, nested amplification with species-specific 16S primers paired with a universal primer located in the 23S gene (L189). The sequence of primers species-specific and L189 were GTC GCT AGA CCG CGA GGT CAT GA (*E. faecalis*/16S/Forward) and GGT ACT TAB ATG TTT CAG TTC (universal primer/23S/Forward), respectively. The PCR reaction conditions were as follows: 27 cycles of 94°C for 1 min., 52°C for 2 min., and 72°C for 3 min. Similarly to the first amplification, positive and negative controls were used in the latter PCR reactions. PCR products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, and viewed under ultraviolet transilluminating light. A positive or negative identification was based on the presence of clear bands of the expected molecular size (831 bp) using a 21-kb lambda DNA ladder (Invitrogen, Carlsbad, CA, USA). All assays were repeated, and if the results were not in agreement, they were repeated again.

Detection of virulence genetic determinants

Virulence genes possibly related to the persistence of *E. faecalis* in endodontic infections were analyzed in the 12 *E. faecalis* strains isolated from root-filled canals with periapical lesions. Whole DNA of *E. faecalis* strains was extracted as previously described. PCR assays were applied targeting the virulence determinants of *efaA*, *ace*, *gelE*, *asa*, *asa373*, *esp*, and *cylA* (SEDGLEY et al., 2005) (Table 1). A 1.5 μL of each DNA

(*E. faecalis* isolated) was mixed with 0.25 μL of each respective primer, 0.5 μL of each deoxyribonucleoside triphosphate (0.2 mm), 1.25 μL of 2 mm $MgCl_2$, 0.125 μL Taq DNA polymerase, 2.5 μL 10X PCR buffer and 18.6 μL nuclease-free water. The PCR conditions were as follows: 2 min. of initial DNA denaturation at 95°C, followed by 35 cycles at 94°C for 20 s, 58°C for 45 s, and 72°C for 1 min. PCR products were separated by electrophoresis using 1.5% agarose gel containing ethidium bromide and analyzed under ultraviolet light.

Table 1. Primers used to detect virulence genes in *Enterococcus faecalis* isolated strains.

Gene	Sequence of primers (5'-3')	Amplicon size (bp)
*efa*A	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	688
ace	GGA ATG ACC GAGAAC GAT GGC GCT TGA TGT TGG CCT GCT TCC G	616
*gel*E	ACC CCG TAT CAT TGG TTT ACG CAT TGC TTT TCC ATC	405
asa	CCA GCC AAC TAT GGC GGA ATC CCT GTC GCA AGA TCG ACTGTA	529
*asa*373	GGACGCACGTACACAAAGCTAC CTGGGTGTGATTCCGCTGTTA	619
esp	TTG CTA ATG CTA GTC CAC GAC C GCG TCA ACA CTT GCA TTG CCG AA	933
*cyl*A	GAC TCG GGG ATT GAT AGG C GCT GCT AAA GCT GCG CTT AC	688

bp, base pairs.

Statistical analysis

Prevalence of *E. faecalis* was recorded as the percentage of cases examined. The effectiveness of culture, traditional PCR and nested PCR to detect *E. faecalis* in clinical samples were also compared. The ability of different techniques to detect *E. faecalis* of the same sample was compared using McNemar's test (SPSS 13.0, SPSS, Chicago, IL) for matched groups. Significance was established at 5% ($p < 0.05$).

Results

Neither microbial growth nor bacterial DNA was observed in any of the sterility check samples.

E. faecalis detection by culture, traditional PCR and nested PCR

Of the 30 root-filled teeth showing periradicular lesion, *E. faecalis* was identified in 7 cases (23.3%) by culture. Two different strains were recovered from 5 canals, comprising 12 strains. Molecular methods identified *E. faecalis* in 13/30 (43.3%) cases by traditional PCR and 23/30 (76.6%) cases by nested PCR. Traditional PCR and nested PCR were significantly more sensitive than culturing in the detection of *E. faecalis* ($p < 0.05$; McNemar's test). All culture positive cases were also PCR and nested PCR positive.

Antibiotic susceptibility

Twelve strains were investigated for antimicrobial susceptibility. The values of MIC_{50} and MIC_{90} refer to the minimal inhibitory concentration that was effective against 50 and 90% of the tested strains. Table 2 also shows the range of MIC for each antibiotic against the *E faecalis* strain tested ($n = 12$), as well as the susceptibility rate of the strains against each antibiotic according to the susceptibility breakpoints previously determined by the NCCLS criteria. All strains were susceptible to amoxicillin, moxifloxacin, vancomycin, benzylpenicillin and amoxicillin-clavulanic acid. Chloramphenicol and ciprofloxacin were effective against 83.3 and 50% of the strains, respectively. About 33% of the isolates were resistant to rifampicin, 16.7% to tetracycline and 8.3% to doxycycline. *E faecalis* strains were resistant to azithromycin (66.7%) and erythromycin (25%).

Virulence genetic determinants

The adherence genes *ace* and *efaA* were observed in all isolates tested. Other virulence determinants, i.e., *gelE*, *asa*, *esp* and *cylA* were determined in 91.6, 83.3, 25, and 16.6% of the strains, respectively. The *asa373* gene was not detected in any strain.

Table 2. Antimicrobial susceptibility of *Enterococcus faecalis* ($n = 12$) strains isolated from root-filled canals to twelve antibiotics.

Antibiotics	MIC (μg mL^{-1})			Susceptibility rate (%)		
	MIC_{50}	MIC_{90}	Range of MIC (μg mL^{-1})	S	I	R
Amoxicillin	0.315	0.75	0.094-1	100.0%	-	-
Rifampicin	2	12	1-32	16.7%	50.0%	33.3%
Moxifloxacin	0.38	1.5	0.094-1.5	100.0%	-	-
Vancomycin	1.5	3	0.25-3	100.0%	-	-
Tetracycline	0.38	24	0.125-32	83.3%	-	16.7%
Ciprofloxacin	1.5	4	0.38-4	50.0%	50.0%	-
Chloramphenicol	6	8	3-12	83.3%	16.7%	-
Benzylpenicillin	1	2	0.75-3	100.0%	-	-
Amoxicillin-Clavulanic Acid	0.5	1	0.25-1	100.0%	-	-
Doxycycline	0.22	12	0.094-16	83.3%	8.3%	8.3%
Erythromycin	2	16	1-16	-	75.0%	25.0%
Azithromycin	12	>256	3->256	-	33.3%	66.7%

*S = susceptible; I = intermediate; R = resistant.

Discussion

Enterococcus *faecalis* in secondary infection

E. faecalis is the most common microorganism associated with treatment failures regardless of the detection method (PINHEIRO et al., 2003a; STUART et al., 2006) because of the following unique characteristics of *E. faecalis*: (a) *Enterococci* are facultative anaerobes, possessing the ability to grow in the presence or absence of oxygen and they survive in very harsh environments including extreme alkaline pH (9.6) and salt concentrations (RÔÇAS et al., 2004); (b) *E. faecalis* has the ability to compete with other microorganisms, invade dentinal tubules and resist nutritional deprivation (STUART et al., 2006); (c) unlike other pathogens, *E. faecalis* can colonize the root canal as the only organism (pure culture) and survive without the support of other bacteria (PINHEIRO et al., 2003a).

Enterococcus *faecalis* detection

In the present study, the occurrence of *E. faecalis* in root-filled teeth associated with periradicular lesions was detected by culture and traditional PCR (23.3 and 43.3%). Other studies found *E. faecalis* ranging from 0 to 70% by culture (GOMES et al., 2004; SCHIRRMEISTER et al., 2007) and 0 to 90% by PCR (SCHIRRMEISTER et al., 2007; GOMES et al., 2008). Rolph et al. (2001) have not found *E. faecalis* in any refractory cases using culture or molecular methods. The differences in findings between the present study and the previously studies may be caused by geographic differences, different dietary intake, variations in clinical sampling and sample analysis methods.

Biochemical test, traditional 16S rDNA PCR and nested PCR demonstrated different results identifying *E. faecalis* in this study. Here, *E. faecalis* was recovered from 7/30, 13/30 and 23/30 root-filled canals examined by culture, traditional PCR and nested PCR analyses, respectively, showing a higher sensitivity of the nested PCR technique over the culture for detecting *E. faecalis* from root-filled canal samples. Our results are in agreement with other studies that have demonstrated that molecular biology methods are more effective than culture in detecting enterococci (SIQUEIRA; RÔÇAS, 2003; SCHIRRMEISTER et al., 2007; GOMES et al., 2008). The reason for this finding possibly relies on the detection limit of both techniques used. The sensitivity of culture is approximately 10^4 to 10^5 cells for target species using nonselective media, while for PCR varies from 10 to 10^2 cells depending on the technique used (SPRATT, 2004). For instance, the nested PCR takes the detection limit down to about 10 cells (SPRATT, 2004; GOMES et al., 2008). PCR can detect nonviable or viable but nonculturable cells (VBNC) (SIGNORETTO et al., 2000). Enterococcus species can remain metabolically active after ceasing to divide, elude growth in vitro under periods of starvation in a form, and later resume growth when conditions become more favorable. To evaluate this bacterial state in clinical samples, they targeted mRNA encoding for pbp5, an enzyme associated with peptidoglycan synthesis and critical for VBNC survival (LLEO et al., 2001). PCR-based detection methods enable rapid identification of both uncultivable and cultivable microbial species with high specificity and sensitivity. Conventional PCR assays, however, detect only the presence or absence, rather than the quantity, of a target microorganism (HEID et al., 1996). Further studies will be done, using quantitative real-time PCR, to quantify the amount of *E. faecalis* in the samples.

Antibiotic susceptibility

The E test method was used in the present study because it provides a simple and a rapid method for quantitative susceptibility testing. Moreover, the MICs obtained with this test are generally in very good agreement with those obtained by agar dilution methods, which is the reference method of the NCCLS (GOMES et al., 2004). It is important to periodically cultivate and test the susceptibility of the strains isolated from the root canals with failure of endodontic therapy, in order to monitor possible changes in the types and antibiotic resistance of microorganisms responsible for failure in endodontic treatment. Antibiotics are often prescribed for the adjunctive treatment of acute endodontic infections, with signs of fever, swelling, lymphadenopathy, trismus or malaise in a healthy patient (RODRIGUEZ-NÚÑEZ et al., 2009). Antibiotics are also more likely to be needed in an immunocompromised patient (BAUMGARTNER; XIA, 2003) or a patient in poor health.

The choice of the antibiotic is usually based on previously published susceptibility testing and previous clinical success (BAUMGARTNER; XIA, 2003). It would be ideal if susceptibility testing could always be undertaken before the prescription of antibiotics. Unfortunately, it usually takes from several days to weeks to cultivate and to do susceptibility tests on bacteria (KHEMALEELAKUL et al., 2002).

All *E. faecalis* strains studied were susceptible to benzylpenicillin, amoxicillin, amoxicillin-clavulanic acid, vancomycin and moxifloxacin in accordance with Pinheiro et al. (2004) and Skucaite et al. (2010).

The MICs of amoxicillin and amoxicillin-clavulanic acid were lower than for benzylpenicillin. These findings are in agreement with previous studies (PINHEIRO et al., 2003b; 2004), which have found that enterococci are more sensitive to amoxicillin than to benzylpenicillin. The results indicated that *E. faecalis* strains isolated from root filled canals with periapical lesions remain susceptible, in vitro, to amoxicillin. However the presence of enterococcal strains resistant to penicillin has been reported in endodontic infections (DAHLÉN et al., 2000), which stresses the need to perform routinely susceptibility tests of these isolates.

The MIC of erythromycin varied between 1 and 16 μg mL^{-1} and resistance was verified in three isolates. Eight strains were found to be resistant to azithromycin (3 to > 256 μg mL^{-1}). The number of resistant strains was higher than values found by Pinheiro et al. (2003b; 2004). In this study, 75% of the isolates showed an intermediate pattern against erythromycin. Similar results were reported by Sedgley et al. (2004) who have found, amongst 12 oral enterococci, eight (66.6%) with an intermediate pattern. Pinheiro et al. (2004) found 28.5% *E. faecalis* susceptible to erythromycin and 14.2% to azithromycin. However, in the present study, none of *E. faecalis* strains were susceptible to erythromycin and azithromycin. Bacterial resistance to these drugs has been increasing over time; which suggests that oral enterococci have become less susceptible. It has been noted that erythromycin is not effective against *E. faecalis*. Kuriyama et al. (2000) have suggested that erythromycin may be effective against mild or moderate infections in people with penicillin allergies, but it may not be suitable in cases of more severe infection. Azythromycin was tested as a surrogate for erythromycin and was found to be less effective against enterococci than erythromycin. Furthermore, the present study showed lower percentage of susceptibility to chloramphenicol, tetracycline and ciprofloxacin when compared with Pinheiro et al. (2004).

Virulence determinants. *efaA* and *ace*: All strains in this study possessed genes *efaA* and *ace* related to the adherence factors, corroborating with Zhu et al. (2010).

The *efaA* gene was previously identified with the use of an antiserum from a patient with *E. faecalis* endocarditis (LOWE et al., 1995). The amino acid sequence of the associated protein, *efaA*, revealed 55 to 60% homology to a group of streptococcal proteins known as adhesins. Thus, it was hypothesized that e*faA* might be functioning as an adhesin in endocarditis. Production of *efaA* by strains of *E. faecalis* is common.

E. faecalis also possesses collagen-binding protein (*ace*), which helps it binds to dentin (HUBBLE et al., 2003). Once available, the starved cells are able to recover by utilizing serum as a nutritional source. Serum, originating from alveolar bone and the periodontal ligament, also helps *E. faecalis* to binds to type I collagen (STUART et al., 2006).

GelE: The expression of virulence factor *gelE* was identified in almost (91.6%) all *E. faecalis* strains isolated from cases of failure of the endodontic treatment. Gelatinase (*gelE*) is a hydrophobic metalloprotease with the capacity of cleaving insulin, casein, hemoglobin, collagen, gelatin and fibrin (WATERS et al., 2003). Studies have been performed in an attempt to associate its proteolytic properties with a higher occurrence of enterococci in endocarditis and bacteremia, urinary infections and oral infections (SEDGLEY et al., 2004). Some studies showed that *gelE* enhances biofilm formation by *E. faecalis* (KRISTICH et al., 2004). Based on in vitro study, biofilm formation and a high expression of *gelE* of *E. faecalis* isolated from the endodontically failed teeth may contribute to the development of apical periodontitis. Furthermore the expression of *gelE* was higher in the biofilm-positive than in biofilm-negative strains ($p < 0.05$) (WANG et al., 2011).

Asa and *Asa373*: The presence of the genes in this study showed a similar pattern to that previously reported for endodontic oral strains (ZHU et al., 2010). *asa* was found in 83.3% of the *E. faecalis* isolates, while *asa373* was not detected in any strain.

Aggregation substance (*asa*) is a pheromone-responsive, plasmid- encoded bacterial adhesin that mediates efficient contact between donor and recipient bacterium, facilitating plasmid exchange. *asa* was also found to mediate binding to extracellular matrix proteins, including type I collagen. Binding to type I collagen by bacteria may be of particular importance with respect to endodontic infections, since this is the main organic component of the dentin (LINDE; GOLDBERG, 1993).

Asa373 differs in its protein structure from the classic *asa* and was reported to exhibit some moderately conserved amino acid motifs, when its database sequence was compared with those of some other bacterial adhesins.

Esp: Coding for surface adhesion was present in 25% of samples in this study. The enterococci surface protein (*esp*) is encoded by the *esp* gene and may be involved in colonization and persistence of *E. faecalis* during infections (SHANKAR et al., 2001). Enterococcal gene *esp*, encoding the high-molecular-weight surface protein *esp*, has been

detected in abundance among bacteremia and endocarditis isolates, but it is rare in stool isolates from healthy individuals (SHANKAR et al., 1999). It is likely that it mediates the primary interaction of the pathogen with host surfaces during biofilm formation (TENDOLKAR et al., 2004).

CylA: In this study, 16.6% of *E. faecalis* strains were determined to carry the *cylA* gene. Cytolysin can induce tissue damage through the lysis of erythrocytes and destruction of host cells. Sedgley et al. (2005) determined 36% of the *E. faecalis* endodontitis-associated strains to be capable of producing hemolysin. The genes in the cyl operon encode cytolysin, where *cylA* is the only reading frame necessary for the expression of the component A, a serine protease.

Conclusion

Strains of *E. faecalis* isolated in root-filled canals showed virulence factors related to adherence. They also showed resistance to some antibiotics commonly used in dentistry.

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