Identification of *Enterococcus faecalis* in Root-filled Teeth With or Without Periradicular Lesions by Culture-dependent and—Independent Approaches

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Abstract

Enterococcus faecalis is the most commonly found species in root-filled teeth evincing recalcitrant periradicular lesions and as a consequence, a role in causation of endodontic treatment failure has been suggested. The purpose of this study was to evaluate the prevalence of this bacterial species in root-filled teeth with or without periradicular lesions. Identification of *E. faecalis* was carried out by polymerase chain reaction (PCR) or conventional culture procedures. Overall, *E. faecalis* was detected by species-specific 16S rRNA gene-based PCR in 40/50 teeth (80%), while culture revealed the occurrence of this species in 8/50 teeth (16%). PCR was significantly more effective than culture in detecting this bacterial species ($p < 0.001$). Of 27 root-filled teeth with no periradicular lesions, *E. faecalis* was found in 22 cases (81.5%) by PCR and in five cases (18.5%) by culture. Of 23 root-filled teeth with periradicular lesions, *E. faecalis* was identified in 18 cases (78%) by PCR and in three cases (13%) by culture. Regardless of the identification technique used, no significant difference was observed when comparing the occurrence of *E. faecalis* in root-filled teeth with and without periradicular lesions ($p > 0.05$). Although these findings apparently put into question the status of *E. faecalis* as the main species causing endodontic treatment failure, other related factors still need to be clarified before this assumption turns into certainty. *(J Endod 2006;32:722–726)*

Key Words

Apical periodontitis, culture, endodontic retreatment, *Enterococcus faecalis*, PCR

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A large body of scientific evidence indicates that microorganisms are the major causative agents of endodontic failure characterized by persistence or appearance of a periradicular inflammatory lesion after treatment (1). Even though extraradicular infections can be responsible for some cases of failed endodontic treatment (2), persistent or secondary intraradicular infections have been the most common findings in cases selected for retreatment because of unsatisfactory outcome (3–8).

Microbiological culturing techniques have been traditionally used to investigate the microbiota associated with endodontic infections and have revealed that *Enterococcus faecalis* is the most frequently found species in persistent/secondary intraradicular infections associated with endodontic treatment failure (3–7). Recently, molecular methods have been used to investigate the microbiota of endodontic infections, and the list of putative pathogens involved with failed endodontic therapy has expanded to include even as-yet-uncultivated bacteria (9, 10). However, in addition to detecting new putative pathogens, molecular biology studies have confirmed the status of *E. faecalis* as the most frequently found species in previously filled root canals that have failed (4, 5). Furthermore, molecular biology methods have strengthened the relationship between persistent/secondary intraradicular infections and endodontic treatment failure, since virtually all examined cases of root-filled teeth with persistent periradicular diseases have been shown to harbor microorganisms (4, 5, 11). Because *E. faecalis* has been found in high prevalence in root-filled teeth with periradicular disease, it has been suggested that this species is involved in the pathogenesis of recalcitrant lesions.

Nevertheless, there are some reports in the literature that have demonstrated that enterococci can also be found in root-filled teeth with no lesions. Engström (12) investigated the occurrence of enterococci in different types of endodontic infections and reported the isolation of these bacteria in 24% of the root-filled teeth with lesions and in 18% of the root-filled teeth with no lesions. Molander et al. (7) examined the microbiological conditions of root-filled teeth and isolated enterococci from 47% of the teeth with periradicular lesions and from 11% of the teeth without lesions. More recently, Kaufman et al. (8) detected enterococci in 6% of the root-filled teeth with lesions and in 23% of the root-filled teeth with no lesions. In addition, some studies have not succeeded in detecting enterococci in root-filled teeth with lesions (9, 13) or have demonstrated that *E. faecalis* is not the dominant species in retreatment cases (11). All of these reports question the involvement of *E. faecalis* in causing endodontic failures. Is this species actually participating in causation of persistent periradicular diseases or is this species only present in the root canal because of its ability to survive in bleak environments like a treated root canal?

Based on these premises, the purpose of this study was to provide additional knowledge of the role of *E. faecalis* in treatment failures by investigating the occurrence of this species in cases of root-filled teeth with or without periradicular disease using two microbiological identification methods—16S rRNA gene-based PCR and culture. The study design also allowed the comparison of the efficacy of these two methods in detecting *E. faecalis* in root-filled teeth.

Materials and Methods

Case Description

Samples were collected from 53 root-filled teeth from consecutive adult patients (ages ranging from 19 to 75 years) who had been referred to the Endodontic Clinic at

two universities (Estácio de Sá University and Federal University of Rio de Janeiro) for root canal retreatment, between the years 2004 and 2005. Twenty-seven teeth had no radiographic evidence of periradicular disease and were referred for endodontic retreatment because of long exposure of the root canal filling material to the oral cavity because of loss of the coronal restoration or when an extensive coronal restoration had to be placed and the technical quality of the endodontic treatment was considered inadequate. The other 26 teeth presented persistent periradicular diseases as revealed by radiographs. Of the teeth included in this study, 11 had no restoration, 9 had temporary or defective permanent restorations, and 33 had adequate permanent restorations. Symptoms were reported for two teeth, one with lesion and the other without lesion. Only one tooth with lesion had a sinus tract. All the root-filled teeth had endodontic therapy completed more than 1 yr previously, and the termini of the root canal fillings ranged from 0 to 5 mm short of the radiographic apex. All teeth showed an absence of periodontal pockets deeper than 4 mm.

Sampling Procedures

All samples were collected by one of the authors. After supragingival plaque removal by scaling, each tooth was cleansed with pumice and isolated with a rubber dam. The tooth and the surrounding field were cleansed with 3% hydrogen peroxide and disinfected with a 2.5% sodium hypochlorite solution. After isolation and disinfection of the operative field, coronal restorations were removed when present. For this purpose, sterile high-speed carbide burs were used until the root filling was exposed. If a post was present, removal was attempted through ultrasonic vibration; if unsuccessful, post removal was performed with a sterile high-speed carbide bur. After completion of the endodontic access, the tooth, clamp, and adjacent rubber dam were once again disinfected with 2.5% NaOCl. For sterility control, two paper points were scrubbed on the disinfected tooth crown and transferred to a tube containing Enterococcosel broth (Becton Dickinson Microbiology Systems, Cockeysville, MD), a selective medium with bile-esculin and sodium azide, and incubated for 72 hours at 35°C.

Coronal gutta-percha was removed by means of sterile Gates Glidden burs, and the apical material was retrieved by using K-type or Hedström files, or both. Removal of root fillings was always performed without the use of chemical solvents. Whenever possible, filling material removed from the canals was transferred to cryotubes containing 1 ml of 5% dimethyl sulfoxide in trypticase-soy broth (Difco, Detroit, MI) (TSB-DMSO). Radiographs were taken to ensure that all filling material had been removed. A small amount of sterile 5% sodium thiosulfate solution was placed into the root canal and a sterile endodontic file was introduced to a level approximately 1 mm short of the tooth apex and a gentle filing motion was applied. The root canal contents were absorbed into at least four paper points. Each paper point was retained in position for one minute. The first and fourth points were transferred to Enterococcosel broth and incubated for 72 h at 35°C. The second and third paper points were transferred to TSB-DMSO and immediately frozen at −20°C.

Culture Procedures

After incubation for 72 hours at 35°C, samples that showed growth in Enterococcosel broth were plated onto trypticase soy agar plates containing 5% sheep blood, and incubated at 37°C for 72 hours. Resulting pure colonies were submitted to conventional tests for identification of *Enterococcus* genus and *E. faecalis* species (14). *E. faecalis* was identified by Gram staining, colony morphology, catalase test, growth in NaCl broth, hydrolysis of esculin in the presence of bile salts, hydrolysis of PYR, and LAP, hydrolysis of arginine, pyruvate utilization, motility, pigmentation production, and carbohydrate fermentation tests (arabinose, mannitol, metil α-D-glucopyranoside, raffinose, sucrose, sorbitol, and sorbose). DNA was also extracted from the isolates and identification was confirmed by PCR using *E. faecalis*-specific primers (see below).

PCR Procedures

Samples in TSB-DMSO were thawed to 37°C for 10 minutes and vortexed for 30 seconds. Paper points were removed from the flasks and the microbial suspension was washed three times with 100 μl of ultrapure water by centrifugation for 2 minutes at 2500 × *g*. Pellets were then resuspended in 100 μl of ultrapure water, boiled for 10 minutes and chilled on ice. After centrifugation to remove cell debris for 10 seconds at 9,000 × *g* at 4°C, the supernatant was collected and used as the template for PCR amplification.

E. faecalis was identified using PCR amplification of signature sequences of the 16S rRNA gene (5). The oligonucleotide species-specific primers for *E. faecalis* were 5′-GTT TAT GCC GCA TGG CAT AAG AG-3′ (forward primer, located at base position 165-187 of the *E. faecalis* 16S rDNA, GenBank accession no. Y18293) and 5′-CCG TCA GGG GAC GTT CAG-3′ (reverse primer, located at base position 457-474 of the *E. faecalis* 16S rDNA, GenBank accession no. Y18293), producing a PCR amplicon of 310 bp.

A pair of universal primers (5′-GAT TAG ATA CCC TGG TAG TCC AC-3′ and 5′-CCC GGG AAC GTA TTC ACC G-3′) that match almost all bacterial 16S rRNA genes at the same position (base positions 786-1387, relative to *Escherichia coli* 16S rRNA gene sequence, accession no. J01695) were used as a positive control for the PCR reaction. It served to indicate the presence of bacteria in the clinical samples. The reported amplicon length is 602 bp (15).

Aliquots of 5 μl of the DNA extracts were used as templates in a PCR reaction specific for detection of *E. faecalis*. PCR amplification was performed in the final volume of the 50 μl containing 1 μM of each species-specific primer, 5 μl of 10X PCR buffer, 2 mM of $MgCl_2$, 1.25 U of *Tth* DNA polymerase (Biotools, Madrid, Spain) and 0.2 mM of each deoxynucleoside triphosphate (Biotools). Cycling parameters for PCR reactions using *E. faecalis*-specific primers or universal primers included an initial denaturation step at 95°C for 2 minutes, followed by 36 cycles of a denaturation step at 95°C for 30 seconds, a primer annealing step at 60°C for 1 minute, an extension step at 72°C for 1 minute and a final step of 72°C for 2 minutes. PCR amplicons were separated by electrophoresis in a 2% agarose gel and visualized on an UV transilluminator.

Data Analysis

Prevalence of *E. faecalis* was recorded as the percentage of cases examined. The χ^2 test with Yates' correction was performed to analyze the association between *E. faecalis* and periradicular disease, taking into consideration the results brought about by PCR or culture. The effectiveness of culture and PCR to detect *E. faecalis* in clinical samples was also compared. PCR results were also utilized to evaluate associations between *E. faecalis* and sex, length of treatment and apical limit of obturation. Significance was established at 5% ($p < 0.05$).

Results

Three cases from root-filled teeth with lesions, two of them with no coronal restoration and the other with an adequate restoration, were excluded from the study because of contamination of the tooth crown by *Enterococcus* species as revealed by the sterility controls. Of the remaining 50 cases, *E. faecalis* was detected by species-specific 16S rRNA gene-based PCR in 40 teeth (80%), while culture procedures revealed the occurrence of this species in eight teeth (16%). PCR was significantly more effective than culture in detecting this bacterial species (p

TABLE 1. *Enterococcus faecalis* Detection in Root-Filled Teeth With or Without Periradicular Lesion by Two Identification Techniques. Results and Patients' Data.

Case	Sex	Age (y)	Tooth	Lesion	Lesion Diameter (in mm)	Length of Treatment (in years)	Apical Limit of the Root Filling	*E. faecalis* by PCR	*E. faecalis* by culture
RN01	Male	44	44	No	-	4	1 mm short	Yes	No
RN04	Female	43	11	No	-	5	1 mm short	No	Yes
RN05	Female	46	24	No	-	5	3 mm short	Yes	No
RN11	Female	40	25	No	-	10	0 mm	Yes	No
RN13	Male	45	11	No	-	5	2 mm short	Yes	No
RN17	Male	45	21	No	-	>5	0 mm	Yes	No
RN18	Male	45	22	No	-	>5	4 mm short	Yes	Yes
RN21	Female	19	25	No	-	3	4 mm short	No	No
RN23	Male	62	44	No	-	12	4 mm short	Yes	No
RN24	Female	46	14	No	-	8	2 mm short	Yes	No
RN26	Male	49	14	No	-	2	1 mm short	Yes	No
RN27	Female	46	23	No	-	5	2 mm short	Yes	No
RN29	Male	26	15	No	-	2	1 mm short	Yes	Yes
RN32	Female	28	21	No	-	2	2 mm short	No	No
RN33	Male	26	25	No	-	>2	0 mm	Yes	No
RN34	Female	33	15	No	-	2	1 mm short	Yes	No
RN38	Male	51	25	No	-	>20	4 mm short	No	No
RN39	Female	36	32	No	-	2	2 mm short	Yes	No
RN41	Female	37	11	No	-	>10	5 mm short	Yes	No
RN43	Male	36	11	No	-	>1	4 mm short	Yes	No
RN44	Female	39	11	No	-	>6	0 mm	Yes	Yes
RN45	Female	45	25	No	-	>5	1 mm short	Yes	Yes
RN47	Male	50	11	No	-	25	3 mm short	Yes	No
RN48	Female	30	15	No	-	2	1 mm short	Yes	No
RN50	Female	29	12	No	-	>5	2 mm short	Yes	No
RN51	Male	26	21	No	-	>1	5 mm short	No	No
RN53	Male	25	22	No	-	>5	1 mm short	Yes	No
RW02	Female	32	21	Yes	1	10	2 mm short	No	No
RW03	Male	22	45	Yes	2	2	1 mm short	Yes	No
RW07	Female	43	12	Yes	12	5	0.5 mm short	Yes	No
RW08	Male	38	12	Yes	11	10	1 mm short	Yes	No
RW09	Female	57	22	Yes	10	2	1 mm short	Yes	No
RW12	Female	28	25	Yes	1	5	1 mm short	Yes	Yes
RW14	Female	41	35	Yes	10	>1	2 mm short	Yes	No
RW15	Female	54	41	Yes	2	10	1 mm short	Yes	No
RW16	Male	62	15	Yes	1	15	1 mm short	No	No
RW19	Male	45	12	Yes	1	>5	0 mm	Yes	No
RW20	Male	52	45	Yes	1	6	3 mm short	Yes	No
RW22	Male	75	41	Yes	6	2	0 mm	No	No
RW25	Female	36	22	Yes	1	11	1 mm short	Yes	No
RW28	Female	30	14	Yes	1	2	2 mm short	Yes	No
RW31	Female	59	22	Yes	3	4	1 mm short	Yes	No
RW35	Male	38	12	Yes	2	6	0 mm	Yes	Yes
RW36	Female	37	34	Yes	1	5	1 mm short	Yes	No
RW37	Male	38	22	Yes	5	4	3 mm short	Yes	Yes
RW40	Female	36	41	Yes	2	2	0 mm	No	No
RW42	Female	39	12	Yes	1	20	2 mm short	No	No
RW46	Female	39	12	Yes	2	3	0 mm	Yes	No
RW49	Female	30	24	Yes	4	2	0 mm	Yes	No
RW52	Female	30	21	Yes	1	3	0.5 mm short	Yes	No

< 0.001). Of the 27 root-filled teeth showing no associated periradicular lesion, *E. faecalis* was found in 22 cases (81.5%) by PCR and in five cases (18.5%) by culture. Of the 23 root-filled teeth showing periradicular bone destruction, *E. faecalis* was identified in 18 cases (78%) by PCR and in three cases (13%) by culture. There was no significant difference when comparing the prevalence of this species in root-filled teeth with or without lesions, regardless of the identification method ($p = 0.94$ for PCR findings and $p = 0.89$ for culture findings). All culture-positive cases were also PCR-positive, except for one sample from a tooth with periradicular lesion. All cases negative for the presence of *E. faecalis* yielded positive results for bacteria, as revealed by PCR using universal 16S rRNA gene primers.

PCR results were also used to check for relationships between *E. faecalis* and other clinical conditions. Overall, *E. faecalis* was found in 23/29 (79%) samples from females and in 17/21 (81%) samples from males, with no significant difference observed ($p = 0.83$). The length of treatment had no apparent influence on the occurrence of *E. faecalis*, with this species being detected by PCR in 17/22 (77%) of the teeth treated less than 5 yr earlier and in 23/28 (82%) teeth whose treatment was performed five or more years earlier ($p = 0.94$). The apical limit of obturation did not influence the prevalence of *E. faecalis* either. Of the 39 teeth treated from 0 to 2 mm short of the root apex, *E. faecalis* was found in 32 (82%), while this species was found in 8/11 (73%) teeth filled more than 2 mm short of the apex ($p = 0.79$). Results are detailed in Table 1.

Discussion

In this study, the presence of *E. faecalis*, a facultative coccus suspected to be associated with failed endodontic therapy, was assessed

in root-filled teeth with or without periradicular lesions by two identification techniques. The study design also allowed a molecular biology approach to be compared with a traditional culturing method with regard to detection of *E. faecalis* in root-filled teeth. The molecular biology method revealed the occurrence of this species in 80% of the cases, while only 16% of the cases were positive after culture identification. The high prevalence of *E. faecalis* in root-filled teeth as revealed by the molecular method with samples freshly collected for this study is in accordance with previous studies using the same PCR protocol for identification of *E. faecalis*. Siqueira and Rôças (5) detected *E. faecalis* in 77% of the root-filled samples from Brazilian patients, while Rôças et al. (4) found this species in 64% of the samples from South Korean patients. However, these findings are not congruent with studies using a different PCR protocol with genus-specific oligonucleotide primers (8, 16). This discrepancy can be related to differences in methodologies but may also have occurred as a result of geographical influence in the composition of the root canal microbiota (17, 18).

Our results are in line with other studies that have demonstrated that molecular biology methods are more effective than culture in detecting enterococci from different sources (19, 20). There are several possible explanations for the higher prevalence values of *E. faecalis* as detected herein by PCR when compared with culture. One possible reason for differences is the ability of molecular methods to detect DNA from dead cells. However, it is highly unlikely that DNA from dead cells can remain intact in a complex background like the infected root canal (21). After cell death, the DNA molecule faces an onslaught of nucleases from other bacteria and fungi in the environment. Other chemical processes, such as oxidation and hydrolysis, can also contribute to DNA damage over time, causing an irreversible loss of nucleotide sequence information.

Therefore, differences are more likely to be related to other factors, such as the higher sensitivity of molecular biology methods, particularly PCR, when compared with culture. Better put, molecular methods can detect fewer cells in a sample. In addition to being more sensitive than culture, molecular biology technology has emerged as a more effective, accurate and reliable means for the identification of bacteria that are difficult to identify by conventional techniques, usually because of an uncommon phenotypic behavior (22, 23), and *E. faecalis* is no exception (24). Interpretation of results from culturing methods is based on characteristics observed in known and reference strains, with predictable biochemical and morphological properties under optimal growth conditions. However, phenotypic characteristics are not static and can change under some circumstances, including stress (25). In addition, phenotypic tests are subject to biases of interpretation (21, 24). Thus, when known microorganisms with uncommon phenotypes are present, reliance on phenotypic characteristics can compromise accurate identification.

Moreover, some strains within a given cultivable species can be uncultivated. One of the reasons for such unculturability can be related to the viable but noncultivable (VBNC) state that some bacteria can develop as a survival strategy when faced with adverse environmental conditions, including starvation (26). In this state, bacteria escape detection by conventional culturing methods as they are unable to grow on either solid or liquid culturing media but are still alive, metabolically active and able to exert pathogenicity (26). VBNC cells can resume active growth when favorable environmental conditions are reestablished. The VBNC state has been described in numerous Gram-negative species, but recently also in *E. faecalis* (27). It has been demonstrated by reverse transcriptase-PCR using the viability indicator mRNA as a target that in adverse environmental conditions enterococcal cells lose their culturability but are capable of maintaining for several months their viability (28). The ability of *E. faecalis* to enter the VBNC state can be one of the reasons for the PCR-positive/culture-negative cases.

All samples positive for culture were also positive for PCR, except for one tooth. The reason for this is most likely the use of different aliquots of sample material for the two methodologies (a much smaller amount of sample was used in PCR). Therefore, because different aliquots of samples may contain variable numbers of target bacteria, this factor may have contributed to the occurrence of this PCR-negative/culture-positive result.

Our results demonstrated that bacteria were found in all cases, including the root-filled teeth with no periradicular lesion. In principle, this finding would come as a surprise. However, it should be pointed out that seven cases from root-filled teeth without lesions had no coronal restoration and 4 other teeth had defective leaking restorations. As a matter of fact, missing or defective coronal restorations represented an indication for retreatment in these teeth. Thus, bacterial presence in several teeth without lesions can be interpreted as a consequence of coronal exposure of the root canal fillings to saliva. Studies have demonstrated that recontamination of the entire extent of treated canals can occur shortly after direct saliva challenge (29, 30). Bacteria penetrating in filled root canals by coronal leakage may establish a secondary intraradicular infection and put the outcome of the treatment at risk (1). In addition, because nine cases with no lesion were poorly treated, bacteria may have been remainders of a primary or secondary infection not eliminated by treatment. Considering the qualitative nature of this study and the high sensitivity of PCR, it is possible that the number of bacterial cells (load) in cases with no lesion may have been low and conceivably below the threshold necessary for a lesion to develop. It is likely that the development of a radiographically detectable periradicular lesion in some of these cases might have been just a matter of time.

For a given microorganism to survive in a filled root canal, it has to resist intracanal procedures of disinfection and endure periods of nutrient deprivation. *E. faecalis* has the ability to penetrate dentinal tubules, sometimes to a deep extent, which can enable this species to escape the effects of intracanal antimicrobial procedures (31). In addition, *E. faecalis* has been shown to be able to form biofilms in root canals, and this ability can be important for bacterial resistance to and persistence after endodontic procedures (32). *E. faecalis* is also resistant to calcium hydroxide, a commonly used intracanal medicament, and such ability to resist high pH values seems to be related to a functioning proton pump, which drives protons into the cell to acidify the cytoplasm (33). Unlike most putative endodontic pathogens that are frequently found in primary infections, *E. faecalis* may colonize root canals in single infections (3). Such a relative independence of living without deriving nutrients from other bacteria can be extremely important for its establishment in filled root canals. Finally, environmental cues can also regulate gene expression in *E. faecalis*, affording this bacterium the ability to adapt to varying environmental conditions and therefore to survive in environments with a scarcity of nutrients and to resume growth when the nutrient source is restored (34, 35). Taken together, all of these properties help explain the significantly high prevalence of *E. faecalis* in root-filled teeth but do not necessarily imply a role for this species in causing persistent periradicular diseases.

In this regard, our findings corroborate those from a previous study in that *E. faecalis* was not associated with root-filled teeth with periradicular disease (8). The present findings, irrespective of the identification method used, apparently puts into question the suspected pathogenetic role of this species in cases of failed endodontic therapy. However, some aspects, including the limitations of this and previous studies, deserve some discussion before a pathogenic role for *E. faecalis* in endodontic failures is dismissed. First, even though radiographs were examined by an experienced endodontist, it is well known that in

some cases a periradicular lesion can be present but remains radiographically undetectable until a given amount of bone is resorbed. Second, it is salient to point out that the protocols used in this study as well as in the study of Kaufman et al. (8) only provide qualitative results. It is possible that the number of *E. faecalis* cells was lower in root-filled teeth without lesions. Thus, one of the requisites for a disease to develop (the bacterial load) may not have been fulfilled or it was just a matter of time. A third factor that deserves some consideration relates to bacterial clonality. It is well known that not all clonal types within a given species are able to cause disease. Therefore, the possibility exists that more virulent clones of *E. faecalis* were present in teeth evincing periradicular diseases. Finally, host resistance can differ from subject to subject and may spark different patterns of response to microbial infection. While all these issues are not properly addressed, a definitive conclusion that *E. faecalis* is not the major pathogen associated with persistent periradicular diseases cannot be drawn.

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