Molecular detection of *Enterococcus* species in root canals of therapy-resistant endodontic infections

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**Objectives.** The objectives were to identify *Enterococcus* spp in nonhealing endodontic cases using PCR amplification and molecular sequencing, and to determine if the prevalence of enterococci is increased in diabetic patients.

**Study design.** Specimens from 40 cases undergoing retreatment were incubated in prereduced thioglycollate broth at 37°C. Extracted DNA had PCR amplification using primers that target the *tuf* gene of 14 *Enterococcus* spp. PCR products were directly sequenced and identified phylogenetically.

**Results.** Three cases were eliminated because the patients were on antibiotics or the tooth did not have a periradicular radiolucency. The remaining 37 specimens included 6 from diabetic patients. Eight specimens were positive for *Enterococcus* spp. Of these, 6 (19%) were from nondiabetic and 2 (33%) from diabetic patients (odds ratio = 2.1; chi squared, *P* = .45). Phylogenetically, all sequences from positive specimens matched *E faecalis* V583 (AE016947).

**Conclusion.** *E faecalis* was the only enterococcal species detected, with an overall prevalence of 22%.


The identification of microorganisms that are capable of resisting endodontic treatment procedures and surviving in the root canal environment would assist in determining effective antimicrobial therapies. These microorganisms could cause failure of treatment, particularly if microscopic pathways to the periradicular region are inadvertently left following obturation. Studies examining the cultivable microbial populations in therapy-resistant endodontic cases have shown a primarily gram-positive bacterial profile. *Enterococcus faecalis* is the most prevalent organism cultured from nonhealing endodontic cases, with a prevalence that ranges from 27% to 56% of cases analyzed.1-6 However, the presence of *E faecalis* in these cases is not a universal finding. An investigation that examined its presence in cases that required retreatment because of nonhealing periradicular lesions, but that had adequate coronal restorations, failed to detect this organism.7

Molecular microbiological techniques may improve the sensitivity of microbial detection compared to culturing8 and therefore enable the identification of enterococci with greater precision. A study that utilized cloning and sequencing of all bacterial DNA based on restriction enzyme patterns in eleven nonhealing endodontic cases failed to detect enterococci.9 However, a more recent study that specifically targeted *E faecalis* with specific 16S rDNA PCR primers identified this organism in 77% of 22 nonhealing cases.10 The wide variability of enterococci reported may be due to the identification technique, geographic differences, or sample size. Furthermore, it is not known if other enterococci are also prevalent in nonhealing endodontic cases.

In a previous study, PCR primers were described that amplify a 112-bp DNA sequence in the *tuf* gene of a number of *Enterococcus* spp.11 The *tuf* gene encodes the Tu elongation factor, which is an essential constituent of the bacterial genome. In that study, these primers correctly identified 159 clinical *Enterococcus* isolates, and were negative with 50 other gram-positive and 44 gram-negative organisms. The primers identified 14 of the 15 *Enterococcus* spp tested, 2 *Abiotrophia* spp, and 4 *Listeria* spp. In another previous study, we used these primers on 24 specimens from primary endodontic infections, and detected enterococci in 3 cases.12

In a clinical outcomes study, we have previously shown that a history of diabetes mellitus was associated with reduced successful outcome of endodontic
treatment in cases with pre-operative periradicular lesions. The fact that treatment outcome was not different between the diabetic and nondiabetic patients when cases with vital pulp were considered indicated that the differences between the 2 groups may be related to the infectious process in the cases with lesions. Diabetes may increase the incidence or severity of common infections such as those occurring in the respiratory or urinary tracts. Additionally, a number of specific infections occur principally in diabetic patients such as foot infections, invasive otitis externa, cholecystitis, pyelonephritis, and rhinocerebral mucormycosis. Interestingly, enterococci are quite prevalent in diabetic foot infections, being present in about 13%-45% of cases and are frequently the primary microorganism or second only to Staphylococcus aureus. It is not known whether the prevalence of Enterococcus spp is increased in nonhealing endodontic cases of diabetic compared with nondiabetic patients.

Therefore, the objectives of this study were to identify Enterococcus spp in root canals of cases with nonhealing periradicular lesions using PCR amplification and molecular sequencing, and to determine if the prevalence of these microorganisms is increased in patients with a history of diabetes mellitus.

MATERIAL AND METHODS
Patient population and clinical specimens
All clinical procedures performed in this study were approved by the Institutional Review Board of the University of Connecticut Health Center and were described in advance to patients, who provided written informed consent. Forty bacterial specimens were obtained aseptically from 37 patients undergoing endodontic retreatment for teeth determined clinically and radiographically to have nonhealing periradicular lesions. The teeth were single-rooted and contained a root filling with associated periradicular lesions. The teeth were single-rooted and contained a root filling with associated periradicular lesions. The age of the patients ranged from 20 to 70 years and the teeth were suitable for rubber dam isolation. The tooth was isolated with a rubber dam, disinfected, and then neutralized according to the protocol by Möller. 

Briefly, the rubber dam, retainer, tooth, and surroundings were disinfected with 30% hydrogen peroxide and 5% tincture of iodine for 2 minutes. The disinfectants were neutralized with 5% sodium thiosulphate, and then a culture of the operating field was taken. Old restorations and caries were removed. A standard access opening was created with a sterile #2 round bur at high speed. The pulp space was accessed and the root filling was mechanically removed with Gates-Glidden burs and hand files with no solvents. A sterile #15 file was placed within the canal and the length adjusted to within 1 mm of the apex with the aid of a Root ZX electronic apex locator. A digital radiograph was exposed to confirm the working length. Sampling fluid was added to the root canal. A sterile #20 file was used to agitate canal contents for 1 minute. The entire canal contents were absorbed onto sterile paper points and transferred to preduced thioglycollate broth. The broth tubes were stored aerobically at 37°C.

An independent endodontist, who was unaware of the microbiological results and who was not involved in the treatment of the patients, examined all preoperative radiographs to determine whether a preoperative lesion that exceeded twice the width of the periodontal ligament space existed. All cases except 1, which was eliminated from the analysis, were determined by this evaluator to have preoperative periradicular lesions. One patient who had 2 teeth retreated required antibiotic premedication, so these 2 cases also were eliminated, leaving a final sample of 37 cases. Six of the patients were type II diabetics, all of them on oral hypoglycemic agents. Four of the 6 diabetics had moderately controlled diabetes, as indicated by a mean (of 3-4 different measurements) fasting blood glucose of 119-144 mg/dL, or an HbA1c of 6.0-6.8. The blood glucose data for 2 diabetics were not available, but verbal assurances were obtained from the patients that their diabetes was under control.

The independent evaluator also used the preoperative radiographs to dichotomize all cases as acceptable or unacceptable on 2 parameters: the previous endodontic obturation and the coronal seal. Acceptable endodontic obturation was defined as the presence of radiopaque material to a level that is 0-2 mm short of the apex, with no voids over 2 mm in diameter anywhere in the filling. Acceptable restoration was defined as the presence of radiopaque coronal restoration sealing the chamber with no recurrent caries.

Microbiological methods
DNA was extracted from the thioglycollate broth specimens that had been incubated aerobically at 37°C. Aliquots were obtained from the broth specimens within 2 weeks of sampling and frozen at −70°C. Eight specimens were incubated for 2.5-7.5 weeks before sampling. For this reason, a pilot experiment was performed in which an ATCC stock strain of E faecalis (19433) was grown in thioglycollate broth and incubated under the same conditions, alone or spiked by an Enterococcus-negative specimen, for 8 weeks. Weekly or biweekly sampling and PCR analysis (see below) revealed consistent positive reaction (data not shown). It was recently shown that E faecalis can survive for 4 months in serum. We further analyzed the survival of E faecalis (19433) in the presence of a mixture of gram-positive and gram-negative bacteria.
with a known nuclease producer to determine if this affects its survival. Thus E faecalis was pooled with ATCC stock strains of Peptostreptococcus micros, Porphyromonas gingivalis, Prevotella intermedia, and Fusobacterium nucleatum, with or without Streptococcus bovis, an oral Streptococcus species that has been shown to produce nucleases.\textsuperscript{22} This pool was incubated and analyzed using PCR at weekly intervals for 6 weeks. Enterococcus PCR reaction remained strongly positive for the duration of the experiment (data not shown). Therefore, the 8 specimens with extended incubation times were analyzed together with the rest of the specimens.

The specimens and control ATCC E faecalis vials were centrifuged for 10 minutes at 7,500 rpm and the supernatant was removed. From the cellular pellet, DNA was extracted using the Qiagen-QIA amp DNA mini kit (Qiagen, Valencia, Calif), according to the manufacturer’s protocol, as described previously.\textsuperscript{12,23} Extraction yielded 400 \(\mu\)L that were aliquoted in sterile DNA- and RNA-free conical tubes and frozen at \(-20^\circ\text{C}\) until use.

The primers that were used to detect Enterococcus sequences\textsuperscript{11} amplify a relatively short DNA fragment of 112 bp. The primers used have the following sequences: forward: TACTGACAAACCATTCATGATG; and reverse: AACTTCGTCACCAACGCGAAC. A previous study has shown that these primers detect the following Enterococcus spp: E avium, E cecorum, E casseliflavus, E dispers, E durans, E faecalis, E faecium, E flavescens, E gallinarum, E hirae, E mundtii, E pseudoavium, E raffinosus, and E saccharolyticus, but not E solitarius, which rarely causes disease in humans.\textsuperscript{11} PCR amplification was performed in a PE9700 thermal cycler (Applied Biosystems, Foster City, Calif). It was carried out in a volume of 50 \(\mu\)L containing 10 \(\mu\)L of extracted specimen DNA or 5 \(\mu\)L of extracted control stock E faecalis DNA, 5 \(\mu\)L of TAE buffer that contains 1.5 mmol/L MgCl\(_2\), 0.5 \(\mu\)L HotStar Taq (Qiagen), 0.2 mmol/L of each of the 4 deoxynucleoside triphosphates (dNTP) (Takara, Otsu, Japan), 0.5 \(\mu\)mol/L (500 ng) of each (forward and reverse) primer and the balance made up of sterile ultrapure water. PCR conditions used were as follows: the initial denaturation was at 94\(^\circ\text{C}\) for 15 minutes followed by 30 cycles of denaturation at 94\(^\circ\text{C}\) for 15 sec, annealing at a 55\(^\circ\text{C}\) for 15 seconds, and extension at 72\(^\circ\text{C}\) for 45 seconds. The final extension was at 72\(^\circ\text{C}\) for 5 minutes, then the products were cooled to 4\(^\circ\text{C}\) until removed. Amplification products were analyzed by 2% agarose gel electrophoresis in tris-acetate EDTA (TAE) buffer (40 mmol/L tris-acetate, 2 mmol/L EDTA, pH 8.3) run at 20 V for 10 minutes followed by 85 V for 150 minutes. They were then stained for 30 minutes with 5 \(\mu\)L of 10 mg/mL ethidium bromide/100 mL buffer; and destained for 15 minutes with water. The PCR products were visualized under UV light using an Alpha Imager (Alpha Innotech, San Leandro, Calif).

The positive control for the PCR reaction was extracted DNA from E faecalis ATCC strain 19433. The negative control included ultrapure water and uninoculated thioglycollate broth. The primers were also previously tested with 18 other endodontic gram-positive and gram-negative pathogens and were found to be negative.\textsuperscript{12}

As an added means of verification, all positive PCR amplicons were directly sequenced to determine the identity of the organism. For sequencing, the PCR products were reamplified and then purified using Marligen Bioscience Rapid PCR Purification System (Marligen Bioscience, Ijamsville, Md). Sequencing reaction mixes were prepared with forward and reverse Enterococcus primers, separately (11-32 ng DNA per reaction mix). Sequencing was performed by the Molecular Core Facility of the University of Connecticut Health Center, using the ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, Calif). Sequences were used to search databases available though NCBI, using the BLAST algorithm, then aligned and phylogenetically analyzed using MacVector 7.2 (Accelrys, San Diego, Calif) as previously described.\textsuperscript{12,23}

**RESULTS**

Using the Enterococcus-specific primers, 8 of 37 nonhealing cases (22\%) were positive for Enterococcus spp (Fig 1). There were no statistical differences in Enterococcus spp prevalence between the group with acceptable preoperative coronal seal (6/30 or 20\%) vs. unacceptable coronal seal (2/7 or 29\%), (odds ratio (OR) = 1.6; chi squared, \(P = .62\)). Likewise, there were no statistical differences between the groups with acceptable (4/18 or 22\%) and unacceptable (4/11 or 36\%) previous obturations (OR = 1.6; chi squared, \(P = .54\)). There was only 1 patient with unacceptable previous obturation and coronal seal, and she did not have Enterococcus spp in the root canal.

Two of the 6 diabetic patients (33\%) were positive for Enterococcus spp, compared with 6 of 31 (19\%). This was a positive association by odds ratio (OR = 2.1) but the differences were not statistically significant (chi squared, \(P = .45\)).

Molecular sequencing of the 8 positive specimens with both forward and reverse primers yielded DNA sequences about 87 bp in length. Alignment of the sequences with their closest matches from a BLAST search was then performed. Phylogenetic analysis revealed that all sequences had their best match with E faecalis V583 (AE016947) (Fig 2). The phylogenetic match of the forward and reverse sequences were
consistent with each other, albeit with slightly different homologies. The homology of the DNA sequences with \( E. faecalis \) V583 ranged from 94% to 100%. The homology percentage was not used in identification because the sequences were on average 90 bp in length, which is very short compared with identifications based on the 16S rDNA (1500 bp in length) for which at least 98% homology is needed for species-level identification.24

**DISCUSSION**

The results of this study show the prevalence of \( E. faecalis \) in nonhealing endodontic cases to be 22%. This finding is different from that recently reported in another molecular study, in which primers that amplify 16S rRNA gene sequences detected this microorganism in 77% of 22 nonhealing cases.10 Because all the enterococci detected in our study were \( E. faecalis \), we repeated the analysis using the primers published in the previous study, to determine if the differences between the 2 studies were related to the sensitivity of the primers. Only 6 of the 8 positive cases were positive with the new primers, and none of the negative cases were positive. Therefore, the differences in findings between the 2 studies are probably mainly due to geographic differences, although they may also be due to differences in coronal leakage or dietary intake in the 2 populations. An older study documented a correlation between the prevalence of enterococci in root canals of primary and retreatment cases and that in other oral sites such as proximal gingival sulcus and tonsils, of the same patients.25 More recently, significantly different prevalence of some endodontic pathogens in primary infections were reported between the Brazilian specimens and those collected by another group in the US.26 These differences could also account for why the prevalence of enterococci in this study is much lower that that reported in a number of culture-based studies.3,5,6 However, as stated before, some studies have failed to show the presence of enterococci in failing cases,7,9 thus the true role of enterococci in causing failure of treatment remains to be determined. It may be that enterococci are opportunistic microorganisms that populate exposed root-filled canals if these microorganisms are present elsewhere in the mouth.

The number of diabetic patients in this study was too low to provide definitive results. Moreover, the diabetic and nondiabetic groups should be similar in many respects for the comparison to be equitable. The variable of diabetes was included in this study for 3 reasons: (1) Diabetics with preoperative lesions are less likely to have successful treatment outcome 2 years postoperatively,13 (2) \( E. faecalis \) has been found to be the most prevalent microorganism in nonhealing cases,1-6 and (3) \( E. faecalis \) is apparently prevalent in diabetic foot infections.15-19 Therefore, the findings in this study were used to conduct a power analysis in order to
Fig 2. Phylogenetic tree of various enterococci and 1 Abiotrophia species, showing the closest matching of the 8 positive specimens with *E. faecalis* (AE016947). The parameters used were neighbor-joining, best tree; Kimura 2-parameter; with the gamma correction off; and the gaps distributed proportionately. The bold numbers indicate bootstrap analysis with 100 replications.
determine the number of diabetic and nondiabetic patients needed to show a significant increase in the prevalence of *E. faecalis* in diabetic patients. Cohen’s *h*1, the effect size for the difference between 2 proportions, is calculated as $h = 2 \arcsin(p_1) - 2 \arcsin(p_2)$, where $p = 2 \arcsin(p)$. Thus, for $p_1 = 0.33$ (prevalence of *E. faecalis* in diabetics) $h = 1.224$, whereas for $p_2 = 0.19$ (prevalence of *E. faecalis* in nondiabetics) $h = 0.902$; therefore, $h = 0.322$, a medium effect size. For a statistical power $(1 - \beta)$ of 0.8, $df = 1$, with an effect size of 0.322, a total sample size of 76 cases would be needed to show statistical significance at $\alpha = .05$.12

In a previous study, primary endodontic infections in 24 cases were analyzed using the same primers and found to have 3 positive specimens.12 We have now completed analysis of 13 additional primary cases, again using the same primers, with no increase in the number of positive specimens. Thus the prevalence of *Enterococcus* spp in these primary treatment cases is 3/37 or 8%. Although this study shows almost a 3-fold increase in *Enterococcus* spp in the failing cases, (OR = 3.13), the differences were not statistically significant (chi squared, $P = .1$). Using the same power analysis method as before shows an effect size of 0.403, and a total sample size of 49 cases needed to show statistically significant differences at statistical power of 0.8 and $\alpha = .05$.

In this study only *E. faecalis* V583 was detected. This is the vancomycin-resistant *Enterococcus* strain for which the entire genome was recently published.28 The high match of sequences analyzed in this study and this particular strain is probably related to the fact that this strain is the most accurately characterized in GenBank at this time. In this study, we did not analyze for the presence of any antibiotic-resistance genes, and therefore could not comment on this aspect of the enterococci detected. A previous study used culture and biochemical analysis to determine the species of enterococci isolated from endodontic infections.29 Twenty-six of the 29 organisms detected were *E. faecalis* and 3 were *E. faecium*. These organisms were found to be resistant to a number of antibiotics but were sensitive to vancomycin. Most culture-based studies, however, found only *E. faecalis* to be present in retreatment cases.3,5,6

The results of this study show that enterococci may not be present in the majority of cases with nonhealing periradicular lesions. More studies are needed to further characterize the role of enterococci in the persistence of periradicular lesions, and to determine their resistance to endodontic disinfection techniques and to antibiotics.

**REFERENCES**


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