Detection and Quantitation of *E. faecalis* by Real-time PCR (qPCR), Reverse Transcription-PCR (RT-PCR), and Cultivation During Endodontic Treatment

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Abstract

Enterococcus faecalis is frequently recovered from refractory endodontic infections and has been implicated in endodontic treatment failures. This study compared real-time quantitative PCR (qPCR) assay to cultivation for *E. faecalis* detection and quantitation during endodontic treatment. A reverse-transcription PCR (RT-PCR) assay was also developed to detect the bacterium clinically in the viable but nonculturable (VBNC) state. Intra-canal samples ($n = 87$) were collected upon access, post-instrumentation/irrigation and postcalcium hydroxide treatment from 15 primary and 14 refractory infections involving 29 single-rooted teeth, and analyzed by the three methods. The bacterium was up to three times more prevalent in refractory than primary infections at each collection step. Overall, qPCR detected significantly more *E. faecalis*-positive teeth and samples than cultivation ($p < 0.001$). VBNC *E. faecalis* was detected by RT-PCR in four samples that were negative by cultivation. These findings support qPCR and RT-PCR as more sensitive methods than cultivation for detecting *E. faecalis* in endodontic infections. (J Endod 2006;32:715–721)

Key Words

Endodontic failure, endodontic treatment, *Enterococcus faecalis*, real-time quantitative, PCR, RT-PCR

Enterococcus faecalis, a Gram-positive facultative anaerobe, is frequently isolated from teeth that fail to heal after root canal treatment. The bacterium represents the predominant species in up to 56% (1) and 77% (2) of refractory endodontic cases using cultivation and standard polymerase chain reaction (PCR), respectively. *E. faecalis* is less common in primary infections, with detection prevalences ranging from 1.6 to 40% by cultivation (3, 4) and 7.5 to 14% by standard PCR (5, 6). Properties that enable the bacterium to persist after therapy include resistance to disinfection by chemomechanical (7, 8) and calcium hydroxide (9) treatments, growth in monoculture (10), and the ability to enter a viable but nonculturable (VBNC) state in response to stress (11, 12).

The role of *E. faecalis* in refractory and primary infections has not been clearly defined. One reason is the use of different methods to detect the bacterium. Cultivation is time-consuming, requires controlled conditions during sampling and transport to ensure microorganism viability, and can lead to variable results based on the experience of the microbiologist (13). In contrast, PCR-based detection methods enable rapid identification of both uncultivable and cultivable microbial species with high specificity and sensitivity (13). Conventional PCR assays, however, detect only the presence or absence, rather than the quantity, of a target microorganism, and cannot distinguish between nonviable and viable microorganisms. Also, standard PCR requires postamplification processing to separate and identify individual PCR products.

Modifications to standard PCR have recently been developed to address these limitations (13). Real-time quantitative PCR (qPCR), such as the *Taq*Man system developed by Applied Biosystems, relies on the release and detection of a fluorescent signal after cleavage of a fluorescent-labeled probe by the 5′-exonuclease activity of *Taq* polymerase. The release of the dye during each amplification round allows the products to be detected and measured in real-time at the cycle when amplification is first detected. References run in parallel with test reactions can be used to estimate the bacterial cell numbers in clinical specimens. qPCR has been used to evaluate *E. faecalis* in samples from oral rinses (14, 15), tongue scrapings (15), and gingival sulcus sites (15) but has been applied to endodontic infections in only one published study to date (14).

Another modification is reverse transcription-PCR (RT-PCR) that uses RNA (in the form of messenger RNA, or mRNA) as the amplification template. A short-lived molecule, mRNA serves as a marker of viability and active replication (16, 17). The ability of certain bacteria such as *E. faecalis* to cause disease yet elude detection by cultivation has led to the concept of the VBNC state (18). In this metabolic state, *E. faecalis* is not cultivable and its presence in infections may be underestimated. Recently, Lleo reported that *E. faecalis* conserves its viability in the VBNC state by limiting protein synthesis to a few key proteins, such as the penicillin binding protein 5 (pbp5) (19). We reasoned that RT-PCR amplification of the pbp5-encoding region of *E. faecalis* mRNA would allow us to detect VBNC *E. faecalis* and, therefore, determine whether endodontic infections contain the bacterium in a VBNC state.

The primary aim of this study was to develop and compare a real-time DNA-based qPCR assay to cultivation for detecting and quantifying *E. faecalis* in clinical endodontic specimens in primary and refractory infections. As a secondary aim, we developed and used an RNA-based RT-PCR assay to detect VBNC *E. faecalis* in clinical specimens. Our

findings will shed light on the contribution of *E. faecalis* to these infection types and may lead to strategies for eliminating this and other pathogens during endodontic therapy.

Materials and Methods

Subject Enrollment

The study protocol was approved by the Institutional Review Board of the UNC School of Dentistry, Chapel Hill, NC. Informed consent and HIPAA release were obtained from all participants before study enrollment.

Subjects who needed endodontic treatment of single-canalled teeth were recruited on a nonrandom, consecutive basis in the Graduate Endodontic Clinics at the University of North Carolina (UNC) School of Dentistry. Exclusion criteria included teeth restored with a temporary restoration, ASA class III or IV health status, antibiotic use in the past month, inability of a subject to complete the study, and age below 18 yr. Study teeth were divided into two groups based on the infection type. Inclusion criteria for primary cases were radiographic evidence of a tooth-associated periapical radiolucent lesion, no response to thermal and electric pulp testing, adequate crown for isolation, and no history of endodontic treatment. Inclusion criteria for refractory cases were a history of endodontic treatment exceeding 5 yr before the study visit, obturation within 5 mm of the apex, radiographic evidence of a periapical radiolucency, adequate restoration, and enough crown for isolation.

Clinical Protocol and Sample Collection

The tooth was anesthetized and isolated with a rubber dam ligated with dental floss. The crown and operating fields were scrubbed thoroughly with 30% hydrogen peroxide. Oraseal putty (Ultradent, South Jordan, UT) was used as a barrier at the junction of the tooth and clamp as needed for complete isolation. All surfaces were coated with tincture of iodine and dried. Random samples were collected from the operating field and tested by culture for sterility.

Endodontic access was obtained with a sterile high-speed carbide bur without water spray until the pulp chamber or root filling was exposed. Post removal was necessary in one case, and was performed as part of the access preparation with rubber dam isolation. Working length was established via radiographs and an apex locator (Root ZX, J. Morita, Irvine, CA). In primary infection cases, sterile orifice shapers (Dentsply/Tulsa Dental, Tulsa, OK) were used and the chamber dried with sterile cotton pellets. Twenty microliters of liquid dental transport medium (LDTM, Anaerobe Systems, Morgan City, CA) was placed into the canal using a sterile tuberculin syringe. The canal was filed with sterile stainless steel K-files to size 20 ISO and to within 1 mm of the estimated working length. LDTM remaining in the canal was absorbed with a sterile paper point and transferred to LDTM medium for microbiological cultivation. A second paper point was inserted into the canal to a depth within 1 mm of working length and transferred to a vial containing 200 μl of Dulbelco's Phosphate Buffered Saline (DPBS) for qPCR analysis. A third paper point was similarly inserted and transferred to a vial containing 200 μl of RNAlater (Ambion, Austin, TX) for RT-PCR analysis. The canal was again filled with LDTM and the sampling procedure was repeated in reverse order using the corresponding vials so that each sampling vial contained two paper points that comprised the Upon Access specimens (S1).

Refractory infection cases were similarly sampled with the following modifications. Gutta-percha was retrieved with orifice shapers and Hedstrom files; no solvent was applied. A sterile K-file one size larger than the last instrument used for gutta-percha removal was placed in the canal and worked with a filing motion to disrupt the canal contents. The file contents were transferred to three S1 sampling vials.

Final shaping and mechanical debridement of the canal was achieved using sterile Pro-File® ISO 0.04 taper nickel-titanium files (Dentsply/Tulsa Dental) in a rotary crown-down technique (Aseptico ITR Electric Torque Control Motor, Dentsply/Tulsa Dental). Teeth were irrigated with 1.05% NaOCl between files and after the final file. The prepared canal was rinsed with 5% sodium thiosulfate followed by sterile saline and dried with sterile paper points. Postinstrumentation and irrigation (S2) specimens were obtained by filling the canal with 20 μl of LDTM, pumping with minimal reaming motion with a sterile file identical to the final instrumentation file, collecting the canal contents onto three successive paper points and transferring the points to sampling vials containing LDTM, DPBS, and RNAlater. The canal was irrigated with NaOCl followed by sterile saline and dried with paper points. A mixture of calcium hydroxide and 0.12% chlorhexidine (PerioGard, Colgate-Palmolive Co., Canton, MA), was placed into canals with a Lentulo spiral filler (Caulk, Milford, Delaware). The teeth were temporized with Fuji IX glass ionomer (Dentsply Int. Inc., York, PA). Specimens collected in LDTM were immediately transported to the UNC Dental Microbiology Laboratory and cultivated as detailed below. Specimens collected in DPBS and RNAlater were frozen at −70°C until processed for nucleic acids.

At the second appointment, scheduled at least 3 wk after the initial appointment, each study tooth was isolated by rubber dam and accessed following the protocol described above. The calcium hydroxide-chlorhexidine dressing was removed with a K-file and sterile saline irrigation, and neutralized with 0.5% citric acid followed by 3% Tween/0.5% L-α-lecithin (20). The canal was irrigated with sterile saline, dried, and sampled with LDTM as detailed above to obtain the post inter-appointment medicament sample (S3). Obturation was completed using gutta-percha and Roth's Sealer (Sultan Chemists, Englewood, NJ), or Resilon and Epiphany sealer (Pentron, Wallingford, CT) using a warm vertical technique.

Specimen Cultivation and Identification

After transport to the Microbiology Laboratory, aliquots of the specimens and random sterility samples were dispersed onto nonselective and selective plates using a spiral plater. A bile, esculin, and azide (BEA) agar plate was used to subculture Enterococcus. If present, black Enterococcus colonies were subjected to a pyrolidonyl-B-napthylamide (PYR) quick test confirming the presence of *E. faecalis* or *faecium*. PYR-positive colonies were then tested for fermentation of manitol and arabinose, with *E. faecalis* presence determined by mannitol fermentation only. The number of colony forming units (cfu) was determined from each specimen and sample. Growth of *E. faecalis* was also confirmed by Gram stain, micromorphology, colony morphology, and selective growth media under aerobic and anaerobic conditions after standard microbiological techniques.

Bacterial Stocks

Pure reference stocks of *E. faecalis* (ATCC 4034) and *E. faecium* (ATCC 4034) were grown in Wilkins-Chalgren broth under anaerobic conditions by standard method. Exponentially growing microorganisms were harvested by centrifugation, washed in PBS and processed immediately for nucleic acids. The stocks were used to develop the *E. faecalis*-specific qPCR and RT-PCR assay, determine the lower level of assay detection, and verify assay specificity for the target bacterium.

DNA Purification

DNAs from bacterial stocks and clinical samples were isolated using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA) according to

the manufacturer's protocol that included lysozyme digestion of cell pellets before RNase and proteinase K treatments. DNA concentration and purity were determined by spectrophotometry. Purified DNAs were stored at $-70°C$ until analysis.

qPCR Conditions

Real-time qPCR amplification and detection were performed with the ABI-PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a 96-well format. To limit contamination, reactions were set-up in a PCR-specific hood in a laboratory that was physically removed from the site of DNA purification, and the reactions were run and analyzed in another laboratory where DNA manipulation was not performed. In triplicate, PCR reactions were performed in a total volume of 50 μl containing 23 μl of template DNA, 1X TaqMan Universal PCR Master Mix (Applied Biosytems), 900 nM each of the *E. faecalis*-specific primers, and 250 nM of the flourogenic probe. The published sequences of the forward and reverse primers and probe were: 5′-CGCTTCTTTCCTCCCGAGT-3′; 5′-GCCATGCGGCATAAACTG-3′; and 5′-CAATTGGAAAGAGGAGTGGCGGACG-3′, respectively (21). qPCR reaction conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Deionized water and DNase-treated *E. faecalis* served as negative controls. Cycle threshold (C_T) values were calculated using the Sequence Detection Software (Applied Biosystems) and compared to an *E. faecalis* standard curve generated in parallel with test samples. *E. faecalis* copy number was calculated from the known mass of *E. faecalis* (3.5 fg/genome). Primer/probe specificity were assessed by using 10-fold [10 to 10^8 copies per μl (cp/μl)] serial dilutions of *E. faecium* DNA as input DNA in the qPCR assay. To evaluate the effect of contaminating DNA, human DNA isolated from frozen peripheral blood mononuclear cells from a healthy donor, was used to serially dilute *E. faecalis* DNA at 1:1 to 1:10^4 ratios), and the DNA mixtures were tested in the qPCR assay.

Development of an *E. faecalis*-Specific RT-PCR Assay

To evaluate whether clinical samples contained *E. faecalis* in the VBNC state, we developed a RT-PCR assay that detected *E. faecalis* mRNA by targeting *pbp5*, a gene that is actively transcribed when the bacterium is in the VBNC form (19). Total RNA was isolated from an exponentially growing pure stock of *E. faecalis* using the RiboPure-Bacteria method (Ambion) and quantified by spectrophometry. RT-PCR was performed on 10-fold dilutions of the purified RNA (5.8 to 0.58 pg) in a single step procedure using the Titan one-tube RT-PCR kit (Roche, Penzberg, Germany). The published sequences of *E. faecalis pbp5*-forward and reverse primers were 5′-GATGCGCAATTAATCGG-3′ and 5′-CATAGCCTGTCGCAAAAC-3′, respectively, and were designed to span introns to prevent amplification of genomic DNA (18). Purified total RNA (17.1 μl) from each specimen was added to 50 μl reactions containing 0.2 mM dNTPs, 5 mM DTT, 5 units RNase Inhibitor, 100 pM *pbp5*-forward primer, 100 pM *pbp5*-reverse primer, 1× RT-PCR reaction buffer, and 1 μl of Titan enzyme. Reverse transcription was performed for 30 min at 60°C and the resulting cDNA was amplified in 40 cycles of denaturation at 94°C for 1.5 min, annealing at 60°C for 1.5 min, and extension at 72°C for 2 min, followed by a final 5 min extension period at 72°C. RT-PCR products were run on 1.25% agarose gels and stained with Vista-Green dye (Covalys, Witterswil, Switzerland). No RT-PCR product was detected in the negative controls that included RNase-treated *E. faecalis* RNA, *E. faecalis* DNA, heat-killed *E. faecalis*, *E. faecium* RNA and sterile water.

RT-PCR Analysis of Clinical Samples

Total RNAs from clinical samples and bacterial stock controls were isolated, subjected to RT-PCR and analyzed by electrophoresis as de-

tailed above. Purified RNAs were stored at $-70°C$ for 2 wk or less before RT-PCR analysis. RT-PCR products not visualized by electrophoresis were subjected to a second 30-cycle round of amplification using 17.1 μl of the first RT-PCR product. Negative controls included RNase-treated *E. faecalis* RNA, *E. faecalis* DNA, DNA from heat-killed *E. faecalis*, and sterile water. Positive controls included *E. faecalis* RNA purified from exponentially growing bacterial stock.

Statistical Analysis

E. faecalis cfu and DNA counts were $\log_{10}$-transformed to normalize the data before analysis. *E. faecalis* titers obtained by qPCR or cultivation were measured in triplicate at each collection time point and are reported as mean ± standard error (SE) for only the samples with measurable *E. faecalis*. Student's *t*-test was used to test for differences in age between infection groups. McNemar's test was used to determine differences between cultivation and qPCR detection of either the numbers of teeth or S1-S2-S3 samples positive for *E. faecalis*. The significance level for all tests was set at $\alpha = 0.05$.

Results

Study Population

From a total of 29 subjects, 15 primary, and 14 refractory infections were included in the study. The primary infection group included four maxillary incisors, three mandibular incisors, five mandibular premolars, and three mandibular canines. The refractory infection group contained seven maxillary incisors, three mandibular incisors, a maxillary canine, a mandibular canine, a maxillary premolar, and a mandibular premolar. No significant difference existed between mean age ± SE in years of the primary and refractory groups (50.8 ± 4.8 versus 53.0 ± 4.6, p > 0.05). The primary group contained eight females and seven males and the refractory group had seven females and seven males. The mean elapsed time since previous endodontic treatment in the refractory group was 23.3 ± 2.3 yr.

Development of the *E. faecalis*-Specific qPCR Assay

Before testing on clinical samples, we used pure stocks of bacterial reference standards to develop and validate the assay, identify its lower detection limit and determine its specificity. DNA was isolated from 10-fold serial dilutions of *E. faecalis* stock containing known bacterial concentrations and amplified in qPCR reactions in two independent experiments. C_T values were determined for the reactions and compared to the known concentrations. Results from a representative experiment are shown in Fig. 1. C_T values of the standards were highly correlated with the calculated concentrations over an eight-log dynamic range (10 to 10^8 copies per μl). The standard curves plotted as C_T versus $\log_{10}$ concentration of each reference stock DNA generated an average linear equation (y = -2.8x + 39.7) that had a very high average goodness of fit (R^2 = 0.99), indicating a reproducible, inversely linear relationship between template DNA numbers and C_T values. The lower detection threshold of the assay was 10 copies/reaction, or 35 fg DNA. The negative controls generated no detectable amplicons. In other control experiments, the assay failed to amplify DNA from the related bacterium, *E. faecium*. In spiking experiments, the presence of human DNA or *E. faecium* DNA in concentrations up to 10^4-fold greater than template DNA had no effect on the detection or quantitation of the *E. faecalis* standards (data not shown). Thus, the qPCR assay is a highly specific and sensitive method for detecting and measuring *E. faecalis*.
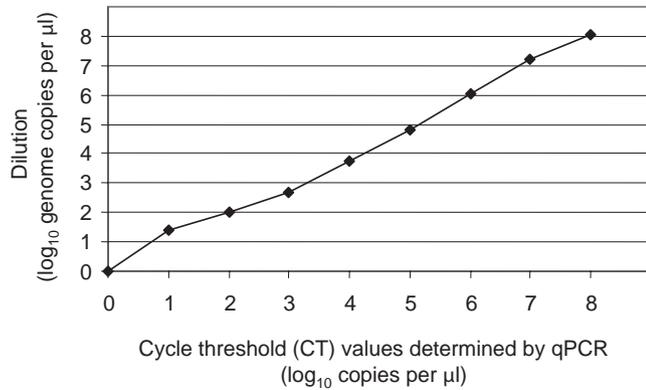
Figure 1. *E. faecalis* standard curve generated by qPCR. DNA from 10-fold dilutions of *E. faecalis* reference stock were purified and amplified by real-time qPCR. Shown is a representative experiment plotting the log10 cycle threshold (C_T) value of each qPCR reaction by its corresponding input $\log_{10}$ DNA concentration.

E. faecalis Detection by Cultivation and qPCR in Clinical Samples

There were 87 intra-canal clinical samples obtained upon access (S1), after instrumentation and irrigation (S2), and after inter-appointment medication (S3) during endodontic treatment of 15 primary and 14 refractory infections from 29 single-canalled teeth. The samples were tested for *E. faecalis* by qPCR and cultivation. Clinical samples yielded qPCR C_T values of 35.8 to 20.4, which corresponded to 10 to 7.9 $\times 10^6$ copies of *E. faecalis* DNA, respectively. The bacterium was identified in three teeth by cultivation and 16 teeth by qPCR (Table 1). The difference in *E. faecalis* detection in any infection type was significantly greater by qPCR than cultivation (p = 0.001). In primary infections, more *E-faecalis* positive teeth were detected by qPCR than cultivation, but the difference did not reach statistical significance. In refractory infections, qPCR detected a significantly more positive teeth than cultivation (10 versus 2 teeth, p < 0.05). When the data were analyzed on a per sample basis, more S1-S2-S3 samples were positive for *E. faecalis* by qPCR than cultivation for primary, refractory, and all infections com-

bined (p = 0.001, <0.05, and 0.001, respectively). qPCR detected all teeth that were positive by cultivation with one exception, as well as 25 samples from 13 culture-negative. All random sterility samples collected from the operating field were negative by culture. These results suggest that *E. faecalis* prevalence is higher in refractory than primary infections and demonstrate that the qPCR assay is more sensitive than cultivation for *E. faecalis* detection.

Persistence of *E. faecalis* During Endodontic Therapy

We then sought to determine the endodontic treatment step at which *E. faecalis* was eliminated from canals (Table 2). In primary infections, *E. faecalis* was present at S1 in 7% (1/15) of cases by cultivation and 13% (2/15) by qPCR. No tooth was positive for the bacterium at either S2 or S3 by cultivation, indicating the removal of culturable *E. faecalis* by the instrumentation + irrigation protocol. Using qPCR, 3 teeth (the two teeth identified at S1 and another tooth) harbored the bacterium at both S2 and S3. This finding may be a result of the loosening of *E. faecalis*-containing dentin during instrumentation and/or bacterial resistance to the disinfection procedure (22).

In refractory infections, *E. faecalis* was found at S1 in 14% (2/14) of cases by cultivation and 43% (6/14) by qPCR. With endodontic therapy, culturable *E. faecalis* was reduced to one case at S2 and totally eliminated at S3. As observed with the primary infections, there was a nonsignificant trend for the number of *E. faecalis* positive cases detected by qPCR to increase to 57% (8/14) at S2 and 50% (7/14) at S3.

qPCR detected up to three times more *E. faecalis* at S1 than cultivation (43% versus 14%), but the difference was not statistically significant. At collection times S2 and S3, qPCR identified more *E. faecalis* infections in refractory lesions than cultivation (p < 0.05). The bacterium was still detected by qPCR in three primary infections and seven refractory infections after treatment (S3).

Effects of Endodontic Treatment on Mean *E. faecalis* Titers

To determine the effectiveness of endodontic therapy on *E. faecalis* titers, we compared $\log_{10}$ *E. faecalis* levels at each treatment step by cultivation and qPCR (Fig. 2). Mean values include *E. faecalis* levels only in teeth that harbored the bacterium, and the number of teeth contributing to each mean is shown in parentheses. In primary infec-

TABLE 1. Detection of *E. faecalis* by Cultivation and qPCR in Endodontic Samples Collected at S1, S2, and S3[a]

Infection Type	No./Unit of Analysis	Detection Method		p-value[b]
		Cultivation No. (%)	qPCR No. (%)	
Total	29 teeth	3 (10%)	16 (55%)	0.001
Primary	15 teeth[c]	1 (7%)	6 (40%)	ns[d]
Refractory	14 teeth	2 (14%)	10 (71%)	<0.05
Total	87 S1, S2, or S3 samples	4 (5%)	29 (33%)	0.001
Primary	45 S1, S2, or S3 samples	1 (2%)	8 (18%)	<0.05
Refractory	42 S1, S2, or S3 samples	3 (7%)	21 (50%)	0.001

[a]S1, upon access; S2, postinstrumentation and irrigation; S3, post inter-appointment medication.

[b]Determined using McNemar's Test.

[c]*E. faecalis* detected in an S1, S2, or S3 sample for each tooth.

[d]ns, not significant.

TABLE 2. Detection of *E. faecalis* by Cultivation and qPCR at Intra-Canal Sample Collection Time Points (S1, S2, and S3) in Endodontic Infections[a]

Method of *E. faecalis* Detection	Primary Infections			Refractory Infections		
	S1	S2	S3	S1	S2	S3
Cultivation	1 (7%)	0 (0%)	0 (0%)	2 (14%)	1 (7%)	0 (0%)
qPCR (DNA)	2 (13%)	3 (20%)	3 (20%)	6 (43%)	8 (57%)	7 (50%)
p-value[a]	ns	Ns	ns	ns	0.025	0.025

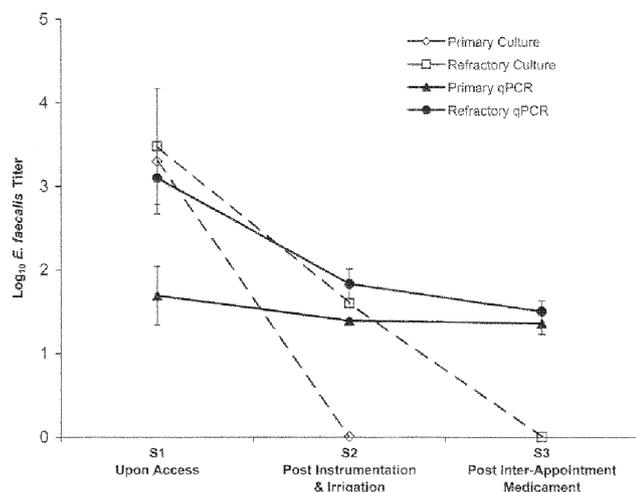[a]p-value determined by McNemar's test; ns, not significant.

Figure 2. Mean $\log_{10}$ *E. faecalis* titers measured by cultivation and qPCR during endodontic treatment. Intra-canal samples were collected at three endodontic treatment steps and assayed for *E. faecalis* by cultivation and real-time qPCR. Shown are the mean $\log_{10} \pm$ SE titers at each collection steps as assayed by the two methods. Only titers from *E. faecalis*-positive samples were used to calculate mean values.

tions, the one *E. faecalis*-positive tooth contained a titer of $\log_{10}$ 3.30 cfu; the bacterium was not detected by this method at S2 or S3. In primary infections, the mean $\pm$ SE levels of *E. faecalis* by qPCR were $\log_{10}$ 1.69 $\pm$ 0.61 ($n = 2$) at S1, 1.39 $\pm$ 0.05 ($n = 3$) at S2, and 1.36 $\pm$ 0.25 ($n = 3$) DNA cp/μl at S3. In refractory infections, mean *E. faecalis* levels by cultivation were $\log_{10}$ 3.48 $\pm$ 1.39 cfu ($n = 2$) at S1 and $\log_{10}$ 1.60 cfu ($n = 1$) at S2; the bacterium was not detected by this method at S3. By qPCR, mean refractory *E. faecalis* DNA levels were $\log_{10}$ 3.10 $\pm$ 0.85 cp/ul ($n = 6$) at S1, 1.83 $\pm$ 0.36 cp/ul ($n = 8$) at S2, and 1.50 $\pm$ 0.25 cp/ul ($n = 7$) at S3. By qPCR, bacterial levels were 40-fold higher by cultivation and 25-fold higher in refractory cases compared to primary cases.

Detection of *E. faecalis* in the VBNC State in Endodontic Infections

To develop and validate the RT-PCR assay, RNA was isolated from serial dilutions of pure *E. faecalis*, subjected the RNAs to RT-PCR and analyzed the products by agarose gel electrophoresis. The anticipated 444 bp band was visualized in reactions containing *E. faecalis* RNA (data not shown). The lower detection limit of the assay was equivalent to 40 cfu (cultivation) or cp/μl of *E. faecalis* DNA (qPCR).

RNAs isolated from the 87 intra-canal samples were then analyzed by RT-PCR. Table 3 lists only those samples that contained *E. faecalis* mRNA detectable by RT-PCR. *E. faecalis* mRNA was detected at S3 in one primary infection and in six refractory infections (S1 only in four teeth, and both S1 and S2 in two teeth). These samples were also positive by qPCR. Only three samples from two teeth (A080 S1 and S2, A083 S1) that were positive by RT-PCR and qPCR were also positive by cultivation. One sample was *E. faecalis*-positive by cultivation but not by RT-PCR or qPCR.

Discussion

Previous investigations of *E. faecalis* in endodontic infections typically used cultivation or traditional PCR to detect the pathogen. Our investigation sought to determine whether more sensitive molecular-based techniques can be used to detect and quantify the bacterium in intra-canal samples obtained from endodontic infections. We found

TABLE 3. Detection of RT-PCR-Positive Endodontic Samples by qPCR and Cultivation

Sample ID (collection time point)	Detection Method		
	RT-PCR[a]	qPCR	Cultivation
A008 (S1)	+	+	−
A057 (S3)	+	+	−
A102 (S1)	+	+	−
A102 (S2)	+	+	−
A080 (S1)	+	+	+
A080 (S2)	+	+	+
A083 (S1)	+	+	+

[a]+, positive; −, negative.

both qPCR and RT-PCR methods to be more sensitive than cultivation for detecting this bacterium in clinical samples.

E. faecalis is the most common, and frequently only, microorganism associated with treatment failures regardless of the detection method (15, 23). In primary infections, the pathogen comprises a small proportion of the total bacterial flora by either cultivation (4, 24) or molecular techniques (5, 6) Although we did not determine the proportion of total bacterial load represented by the bacterium, our study demonstrated its presence in 2 to 3 times as many teeth upon access (S1) in refractory than primary infections by cultivation and qPCR (Table 2).

The qPCR assay detected significantly more teeth and more S1-S3 samples with *E. faecalis* than cultivation in both infection types. Every culture-positive sample was also qPCR-positive with one exception, despite the fact all samples were collected in the order of cultivation-DNA-RNA, biasing the results in favor of cultivation. That is, the first paper point used to sample the canal was designated for cultivation analysis. This sequence maintained consistent sampling between time points and subjects, and provided cultivation (the current gold-standard) with the greatest chance at recovering the bacterium. The reason for detection by cultivation but not qPCR and RT-PCR in the one sample is not known, but may be because of an *E. faecalis* strain that is not recognized by the highly specific molecular methods.

In primary infections, cultivation studies have reported *E. faecalis* detection prevalences of 1.6 to 40% (3, 4, 15, 24). Using molecular methods, the reported prevalences in this infection type were 7.5 to 15% by standard PCR (5, 6), 7.5% by checkerboard hybridization (24), and 11% by genotyping (6). Our prevalence findings of 7% and 13% of *E. faecalis* by cultivation and qPCR, respectively, are consistent with these published results.

The prevalence of *E. faecalis* in refractory infections by cultivation has been estimated at 16.6% (9) to 35% (25) of teeth sampled. Our reported prevalence of 14.3% of teeth sampled at S1 is relatively low and may reflect differences in sample sizes, cultivation methods, and inclusion of multi-canalled teeth in the various studies.

Molecular analyses supporting *E. faecalis* as the predominant species in refractory cases are less extensive than cultivation studies. Rocas et al. (26) found *E. faecalis* in 64% of refractory cases in a North Korean population, while Siquiera et al. (2) identified the bacterium in 77% of refractory cases in a Brazilian population. Recently, Fouad et al. (27) detected *E. faecalis* in 22% of 37 refractory cases using standard PCR. Our detection of the bacterium in 47% of refractory infections upon endodontic access is consistent with these reports.

Cultivation showed that the instrumentation/irrigation protocol eliminated viable *E. faecalis* from primary infections and decreased the number of *E. faecalis*-positive teeth in 1 of 2 (50%) refractory infections. As evaluated by qPCR, however, the treatment had little effect on reducing *E. faecalis* prevalence. This finding may be due the release of

E. faecalis DNA from dentinal tubules during instrumentation (28). The use of large apical file sizes in our study may have removed more infected dentin and allowed irrigation to be more effective compared to other studies. Alternatively, the result may reflect the resistance of the bacterium to disinfection (7, 8, 10, 22). Although the protocol reduced *E. faecalis* levels, the bacterium was not totally eliminated from the treated canals, as evidenced by *E. faecalis* DNA and RNA detected by qPCR and RT-PCR, respectively, at S2 and S3 in primary and refractory infections.

The calcium hydroxide-chlorhexidine dressing present during the inter-appointment step (S3) led to a further decrease in *E. faecalis* DNA in both infection groups. This result may be because of the fairly low quantity of target DNA that was present in treated canals. In an in vitro study, Fouad and Barry (30) reported that pretreatment with 2% chlorhexidine of *E. faecalis* cultures (10^5 cells/ml) did not interfere with DNA detection by PCR but resulted in DNA that was detectable by PCR but not cultivation. Cultures that were grown at less than 10^5 cells/ml and similarly treated were undetectable by both methods, suggesting that relatively high quantities of *E. faecalis* (e.g. $>10^5$ cell/ml) may be required to prevent interference of bacterial assays in the presence of chlorhexidine. However, the 2% chlorhexidine concentration used in the Fouad and Barry study is nearly 17 times stronger than the 0.12% concentration we used. Furthermore, the finding may also be because of irrigation before sampling or the hydrolysis of target DNA and/or RNA by free hydroxyl ions. Among the refractory infections, one tooth containing viable *E. faecalis* at S2 no longer had culturable *E. faecalis* at S3, supporting a report that the chlorhexidine-containing dressing can kill *E. faecalis* in culture (29). Additional studies are needed to substantiate the effects of the combined calcium hydroxide-chlorhexidine medicament on treatment success and *E. faecalis* viability and titer.

By RT-PCR, we detected *E. faecalis* in seven samples that were negative by cultivation, suggesting that the bacterium was in the VBNC form. This finding supports previous studies of VBNC *E. faecalis* in environmental water (19) and a laboratory microcosm (31). Enterococcal species can remain metabolically active after ceasing to divide, elude growth in vitro under periods of starvation in a VBNC form, and later resume growth when conditions become more favorable (11). To evaluate this bacterial state in clinical samples, we targeted mRNA encoding for pbp5, an enzyme associated with peptidoglycan synthesis and critical for VBNC survival (19, 31). mRNA is highly labile because of rapid degradation by endogenous nucleases. mRNA detection by RT-PCR suggests that even when undetected by cultivation, *E. faecalis* remains viable and may return to an active, pathogenic state (31). The period of viable inactivity and eventual resuscitation has particular relevance to endodontics. In a study of *E. faecalis* starvation, Figdor et al. (32) showed that after an initial rapid decline in cell numbers, a small proportion of *E. faecalis* could survive in water and nutrient-limited media (e.g. 1% human serum) for several months. Nutrition to sustain the microbial flora in filled root canals comes from fluid within dentinal tubules and periapical tissues (9, 12). This fluid contains a serum-like substance derived from surrounding bone and connective tissue, and might support growth of selected microorganisms (33). We speculate that the combination of viable *E. faecalis* and a serum-based nutritional source might allow the bacterium to perpetuate the cycle of colonization, canal infection and apical periodontitis despite disinfection, obturation, and sealing of the canal.

Alternatively, viable bacteria might be present at numbers small enough to be detected by the more sensitive molecular methods but not by cultivation. In support of this explanation, every sample that was positive by the RNA-based RT-PCR assay was also positive by the DNA-based qPCR assay. Also, the average duration since previous endodontic treatment of samples exceeded 23 yr. Further investigations using mRNA

analysis to evaluate the endodontic microflora are needed to resolve this apparent discrepancy.

A shortcoming of our study is that samples from the operating field were assessed for culture sterility but not for PCR contamination. Ng and colleagues (34) showed that DNA from dead bacteria can be reduced significantly by washing the tooth surface with a 2.5% sodium hypochloric solution, compared to the 10% iodine solution that was used in our study. It is possible that contaminating DNA from the tooth surface remained intact after endodontic access and gutta-percha removal. DNAse I treatment of RNA preparation before RT-PCR analysis, however, has been shown to effectively remove contaminating DNA (35). The high detection correlation of *E. faecalis* DNA and RNA the highly sensitive molecular techniques, the use of DNAse I pretreatment of RNA preparations, and the negative results from multiple assay controls suggest the absence of PCR contamination in our study.

In summary, we show that qPCR and RT-PCR techniques are more sensitive than traditional cultivation in detecting and quantifying *E. faecalis* in endodontic infections. We also demonstrate that the bacterium, likely in the VBNC state, can persist after therapy. Further studies are needed to delineate the contribution of the bacterium relative to the total microflora in the infection types and to develop more effective methods for eliminating bacteria during endodontic treatment.

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