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Original

Isolation and identification of *Enterococcus faecalis* from necrotic root canals using multiplex PCR

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Abstract: This study was designed to survey the incidence of *Enterococcus faecalis* infection in symptomatic and asymptomatic root canals of necrotic teeth using PCR and to isolate the bacterium for further screening. Sixty patients categorized according to their clinical symptoms were used for sampling by insertion of paper points into the root canals and absorbing all the fluids present within them. The samples were incubated in 1.0 ml 2xYT (containing 16 g bacto tryptone, 10 g yeast extract and 5.0 g NaCl per liter) for 24 h at 37°C without aeration prior to multiplex PCR analysis. To assist the isolation of *E. faecalis*, subsamples were further grown in the same medium supplemented with 6.5% NaCl and back-inoculated into bile esculin. Using multiple cultivation-dependent and PCR analyses, 6 cases (10%) of *E. faecalis* were identified. Four isolates were obtained from asymptomatic cases of chronic apical periodontitis, and the other two were associated with phoenix abscess and acute apical abscess, respectively. No *E. faecalis* infection was found in 5 patients with acute apical periodontitis or in 9 with chronic suppurative periodontitis. Our results indicate that there is no significant difference in the incidence of *E. faecalis* between symptomatic and asymptomatic necrotic dental root canals ($P > 0.05$). (J. Oral Sci. 49, 221-227, 2007)

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Introduction

Infections of endodontic origin are produced by microorganisms that gain access to the normally sterile pulpal and periapical tissues (1). *Enterococcus faecalis* is a non-spore-forming fermentative, facultatively anaerobic Gram-positive coccus. It is an opportunistic pathogen associated with oral infections including marginal periodontitis, infected root canals and periradicular abscesses (2-5). Its association with treatment failure in root-filled teeth is reported to be significant (2). In addition to *E. faecalis*, several other bacterial pathogens including *Peptostreptococcus micros*, *Porphyromonas gingivalis/endodontalis*, *Fusobacterium necrophorum/nucleatum*, *Prevotella intermedia/nigrescens*, *Tannerella forsythia* (*Bacteroides forsythus*), *Streptococcus* species, and also *Candida albicans* have been reported to be involved in mixed infections of necrotic root canals and other periradicular lesions (3,5-8).

The possible role or association of these bacteria in dental infections has been suggested, however, the pathogenicity of each species could not be fully determined (9-13). Despite having limited ability to compete in primary infections, *E. faecalis* has been shown to be more persistent in chronic and asymptomatic infections. Its durability in response to disinfection agents and mechanisms of long-term survival in adverse conditions have been noted (2).

Despite the bacterial diversity seen in necrotic root canals, the pathogens involved in failure of root therapy have been shown to be limited to a few species with significant prevalence of *E. faecalis* (2).

Detection and identification of microorganisms involved in endondotic infection using cultivation-based techniques has been a challenging task due to the mixed nature of the infections and the diverse physiological and nutritional requirements for culture. PCR-based diagnosis of *E. faecalis* has been reported by many workers (14-21).

Variations in clinical sampling and methods of sample analysis have led to differences in the reported prevalence of *E. faecalis* in root canals (6). Culture techniques and molecular-based methods have yielded different results in this respect (20,21), although the variation may also be partly attributable to geographical location (4).

This report describes the results of a survey of *E. faecalis* infection in necrotic root canals and isolation of the pathogen for further studies. To investigate the possible correlation of *E. faecalis* infection with specific signs and symptoms, the studied cases were categorized according to the guidelines described in Pathways of the Pulp (22). This survey was made possible by establishing procedures for multiplex PCR detection of *E. faecalis*, its isolation, and identification using a classic microbiology approach, and finally examining the data by chi-squared test. As a long-term objective of this study, we will screen for treatment failure among patients with root-filled teeth, with or without *E. faecalis* infection.

Materials and Methods

Sample source

Microbial samples of necrotic root canals were taken from 60 patients (ranging in age from 10 to 61 years, including 26 males and 34 females) referred to the Endodontic Ward of Tabriz University of Medical Sciences. The diagnostic procedure to confirm necrotic canals or periradicular lesions involved pulp vitality tests and radiography, and the samples were grouped according to the clinical diagnosis as described in (22).

All of the teeth had accompanying carious lesions, necrotic pulp, periodontal pockets not deeper than 4 mm and no significant gingival recession. Furthermore, teeth with acute apical abscess and phoenix abscess were clinically identified by swelling and pain on biting pressure, percussion and palpation. These groups were differentiated on the basis of X-ray radiography. While only widening of the periodontal ligament was seen in cases of acute apical abscess, phoenix abscesses were accompanied by marked periradicular lucency. No clinical symptoms or draining sinus tracts were seen in patients with chronic apical periodontitis. Cases of chronic suppurative periodontitis were differentiated from chronic apical periododontitis on the basis of whether the sinus tracts originating from chronic endodontic infection were associated with drainage to the gingival surface.

Patients who had taken antibiotics within the last 3 months were excluded. All patients provided signed informed consent. In the case of young patients, informed consent was obtained from their parents/guardians. Additionally, 53 microbial samples collected for different purposes from the gingival sulcus of 27 patients diagnosed as having aggressive periodontitis were used as controls for detection of *E. faecalis*.

Sampling procedure

In collecting the microbial samples, care was taken to avoid cross-contamination. Each tooth was cleaned with a slurry of pumice (Pumex SpA, Milan, Italy) and isolated with a rubber dam (Coltene/Whaledent, Mahwah, NJ, USA). Access preparations were made using sterile burs (D&Z, Wiesbaden, Germany) without a water spray. A K-type file (Maillefer, Ballaiques, Switzerland) was introduced to a level approximately 1.0 mm below the radiographic apex, and filing to #30 was carried out in each canal without any irrigation. If the root canal was dry, a small amount of sterile saline was introduced into it. Two paper points (Diadent, Chongju City, Korea) were then inserted into the canal for 1.0 min to soak up the fluid. The paper points were immediately transferred to microcentrifuge tubes containing 1.0 ml 2xYT medium [containing 16 g bacto tryptone (Scharau, Barcelona, Spain), 10 g yeast extract (Scharau) and 5.0 g sodium chloride (Merck, Darmstadt, Germany) in 1 liter].

Bacterial propagation and isolation

Samples inoculated into culture media were incubated for 24 h at 37°C with no agitation prior to multiplex PCR analysis. These samples were used to re-inoculate 1.0 ml of liquid 2xYT supplemented with 6.5% sodium chloride and incubated for 1-3 days under the same conditions. Cultures showing turbidity were further inoculated into microcentrifuge tubes containing 0.5 ml of bile esculin agar (Scharau). In cases of bile esculin hydrolysis associated with black pigmentation, attempts were made to isolate *E. faecalis* using inocula from cultures grown in 2xYT or on bile esculin agar. Single bacterial colonies were obtained on blood agar (containing 5% fresh sheep blood) and phenyl ethyl alcohol agar (PEA). Furthermore, to confirm the identities of *E. faecalis* isolates, they were examined by multiple physiological assays (23,24). All bacterial cultures, as well as any isolated colonies, were tested by

PCR.

PCR analysis

On the basis of previous reports (14,16) and genetic databases, three pairs of primers were designed for identification and detection of *E. faecalis* (Table 1). All the primers were designed according to the full-length sequence of *E. faecalis* V583, and were synthesized by MWG, Germany. To prepare the PCR template, cells were harvested from 1.0 ml of liquid culture by centrifugation for 1 min at 8000 × *g* (Eppendorf 5415D, Hamburg, Germany). The bacterial pellet was resuspended in 100 µl of 1.0× PCR buffer or its close equivalent [Tris (Merck, Darmstadt, Germany) 10 mM, and KCl (Merck) 50 mM, pH 8.0]. The suspension was heated for 15 min at 95°C and clarified by centrifugation for 1 min at 8000 × *g*. The supernatant was stored at -20°C until used for PCR.

Reaction conditions were optimized by multiple analyses using an *E. faecalis* standard isolate (ATCC 29212) as a positive control. Gradient PCR (Eppendorf, Hamburg, Germany) was used to determine the optimal range of annealing temperature. The reaction cocktail contained 1.0× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, Vilnius, Lithuania), 6 primers each at 0.5 µM, and 2 units of recombinant *Taq* DNA polymerase (Cinnagen Inc., Tehran, Iran). The final volume was adjusted to 25 µl per reaction including 2-5 µl of template. The reaction conditions included a lid temperature of 105°C along with 4 min of initial denaturation at 95°C followed by 35 cycles of 95°C/30 s, 58°C/30 s and 72°C/1.5 min. The reactions were ended by additional extension at 72°C/10 min.

Gel electrophoresis

The entire PCR products were loaded into 1% agarose (Cinnagen, Tehran, Iran) gel and electrophoresed (Akhtarian, Tehran, Iran) for 1-1.5 h in 1× TAE buffer along with a molecular weight marker. After staining the gel with ethidium bromide (Merck) solution, the DNA bands were visualized under UV illumination (UVP Gel Documentation, Upland, CA, USA). Samples containing genomic DNA of *E. faecalis* produced three fragments of 1522, 803, and 650 base pairs (bp), while a ~ 1500-bp band was seen in samples lacking *E. faecalis*. The chi-squared test was used to examine the significance of data.

Results

Analysis of the gel obtained by gradient multiplex PCR is demonstrated in Fig. 1. As can be seen, three fragments of 650, 803, and 1522 bp (corresponding to genomic data) were amplified using three pairs of primers and the genomic DNA of *E. faecalis* as a template. The reactions were

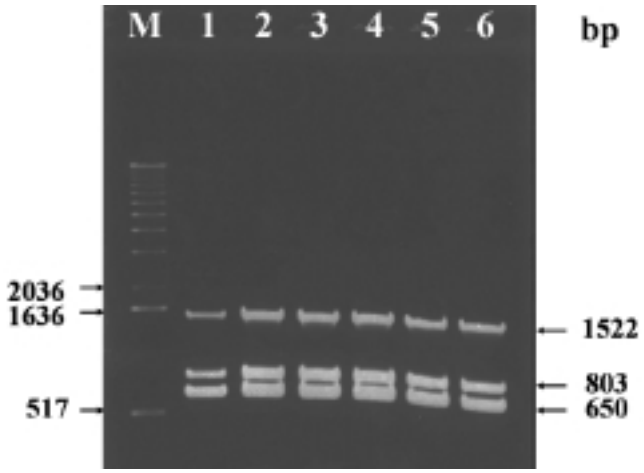

Fig. 1 Gradient multiplex-PCR on 1% agarose gel stained with EtBr, along with molecular weight marker (lane 'M') and corresponding fragment sizes shown on the left-hand side. PCR was conducted over a broad range of annealing temperature from 50 to 60°C (lanes 1 through 6). Numbers on the right-hand side correspond to the fragment sizes of PCR products from *E. faecalis*.

Table 1 Primers designed and utilized in this study to identify and detect *Enterococcus faecalis*

Primer Name	Primer Sequence and Genomic Position	Binding Spec.	Frag. size	Targeting Site
Ef16F	5'-AGAGTTTGATCCTGGCTCA-3' (positioned at 248466-83)	Semi-specific	1522 bp	Full-length coding sequence of 16S ribosomal RNA (4× per genome)
Ef16R	5'-GGTTACCTTGTTACGACTTC-3' (positioned at 249987-68)			
EfisF	5'-ATGCCGACATTGAAAGAAAAAATT-3' (positioned at 300261-84)	specific	803 bp	coding region of iron-sulfur binding protein
EfisR	5'-TCAATCTTTGGTTCCATCTCT-3' (positioned at 301063-43)			
EfesF	5'-GTGTTAAAACCATTAGGCGAT-3' (positioned at 112289-69)	specific	650 bp	coding region of GroES/EL chaperone protein
EfgsR	5'-AAGCCTTCACGAACAATGG-3' (positioned at 111640-58)			

performed optimally over a broad range of annealing temperature from 50 to 60°C, and showed no background or non-specific bands. PCR analysis of representative clinical samples and bacterial cultures is shown in Fig. 2. Lanes with three bands indicated the presence of *E. faecalis* in the sample. Lanes with a ~1500-bp band alone indicated absence of *E. faecalis* and presence of other bacteria. Samples lacking *E. faecalis* were almost unable to grow in medium supplemented with 6.5% NaCl, and hence no fragment was amplified.

In identification of *E. faecalis*, similar results were observed consistently through both cultivation and cultivation-based PCR. All microbial samples lacking *E. faecalis* and all pure clinical isolates (not listed) produced a ~1500-bp fragment corresponding to the 16S rRNA gene. However, after culturing these samples in medium supplemented with 6.5% NaCl, the ~1500-bp fragment was not amplified. A survey of 53 samples collected from 27 patients diagnosed as having aggressive periodontitis used as a control identified no *E. faecalis* through cultivation-based PCR.

The clinical data, along with the results of cultivation and culture-dependent multiplex PCR confirming the status of *E. faecalis* infection, are described in detail in Table 2 and further summarized in Table 3. The results revealed that the incidence of *E. faecalis* infection was 10% (6 out of 60). This included 4 cases (10.5%) of *E. faecalis* in 38 asymptomatic patients (diagnosed as having chronic apical periodontitis) and 2 cases (9.09%) in 22 symptomatic patients, including one case of acute apical abscess and one case of phoenix abscess. Analysis by chi-square tests revealed no relationship between clinical signs and

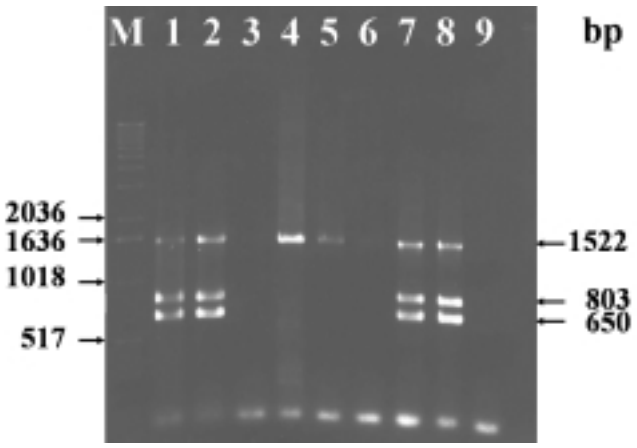

Fig. 2 Agarose gel electrophoresis of multiplex PCR products from clinical samples taken from necrotic root canals. Lane 'M' represents the molecular weight marker, with corresponding fragment sizes on the left-hand side. Lanes 1, 2, 7, and 8 represent *E. faecalis* infection. In lanes 4 and 5, amplification of the 16S rRNA gene (internal control) detects bacteria other than *E. faecalis*. No amplification is seen in lanes 3 and 6 for samples grown in the presence of 6.5% NaCl. Lane 9 is a PCR negative control. Numbers on the right-hand side of the figure correspond to the fragment sizes of the PCR products.

Table 2 Supplementary table detailing the source of each sample along with clinical diagnosis and the results of cultivation and culture-dependent multiplex PCR confirming the status of *E. faecalis* infection

Case No.	Age	Gender	Diagnosis	*E. faecalis* infection	Tooth No.*
1	10	F	1	N	30
2	48	M	3	N	17
3	28	F	3	N	19
4	19	M	1	N	19
5	30	F	2	N	25
6	17	F	2	N	15
7	52	M	3	N	18
8	**21**	**M**	**3**	**Y**	**18**
9	**45**	**F**	**1**	**Y**	**21**
10	15	F	3	N	9
11	13	M	3	N	19
12	16	M	4	N	30
13	20	F	3	N	30
14	14	F	4	N	4
15	21	M	3	N	30
16	38	F	4	N	12
17	61	M	3	N	27
18	34	F	3	N	14
19	20	F	3	N	30
20	**40**	**F**	**3**	**Y**	**20**
21	39	M	3	N	19
22	**18**	**M**	**5**	**Y**	**9**
23	21	M	4	N	19
24	14	F	4	N	30
25	21	M	3	N	9
26	**37**	**F**	**3**	**Y**	**18**
27	25	F	3	N	12
28	52	M	4	N	19
29	39	F	2	N	31
30	18	F	3	N	4
31	37	F	3	N	12
32	51	F	3	N	6
33	53	M	3	N	12
34	32	M	3	N	10
35	52	F	4	N	9
36	19	F	3	N	29
37	43	F	4	N	18
38	23	M	3	N	5
39	54	F	3	N	13
40	18	M	3	N	10
41	49	M	2	N	3
42	**59**	**M**	**3**	**Y**	**8**
43	13	M	3	N	4
44	24	F	3	N	14
45	26	F	3	N	13
46	44	M	3	N	2
47	57	F	3	N	2
48	60	M	3	N	29
49	47	M	3	N	23
50	47	M	1	N	28
51	52	F	2	N	29
52	61	M	4	N	2
53	33	F	3	N	12
54	46	F	3	N	6
55	25	F	1	N	13
56	19	F	3	N	30
57	53	M	5	N	30
58	23	F	1	N	25
59	43	F	3	N	26
60	16	F	3	N	14

Diagnosis:
1. Acute apical abscess
2. Acute apical Periodontitis
3. Chronic apical periodontitis
4. Chronic supporative periodontitis
5. Phoenix abscess
* Tooth numbering was based on universal system

Table 3 Summarized clinical and laboratory data, including the number of cases in each diagnostic group along with the results of cultivation and culture-dependent multiplex PCR confirming the status of *E. faecalis* infection

Patient Diagnosis	Symptomatic	Number of cases	Incidence of *E. faecalis*
Acute apical abscess	Yes	6	**1**
Acute apical periodontitis	Yes	5	0
Chronic suppurative periodontitis	Yes	9	0
Phoenix abscess	Yes	2	**1**
Chronic apical periodontitis	No	38	**4 (10.5%)**
Total		**60**	**6 (10%)**

symptoms and the presence of *E. faecalis* in necrotic dental root canals ($P > 0.05$).

Discussion

PCR amplification of a species-specific DNA sequence enables precise identification of known microbial species and the detection of microorganisms that cannot be cultivated or readily diagnosed (4). Cultivation-based multiplex PCR was used in this study to produce higher specificity for isolation and diagnosis of *E. faecalis*. Additional experiments were also conducted to collect pure isolates of *E. faecalis*, which might represent rare strains of this bacterium from dental root canals, at least in the Iranian community. Simple steps for additional culture in the presence of excess sodium chloride (6.5%) and testing for hydrolysis of bile esculin enriched the bacterial pool in such a way that most of the colonies present on subsequent plates proved to be *E. faecalis*.

Unlike many anaerobic bacteria reported in the oral cavity that cannot be detected through cultivation methods, the present approach was shown to have great flexibility and simplicity for cultivation and isolation of *E. faecalis*. In fact the detection limit of cultivation-based PCR was improved by initial culturing of the clinical samples. Recent reports have indicated the superiority of PCR, quantitative real time PCR and reverse transcription PCR over cultivation for detection of *E. faecalis*, as the latter often produces false negative results (20,21,25).

The reaction conditions using specially prepared primers (Table 1) were optimized by multiple analyses, and the most appropriate reaction conditions were applied in our survey. All of the primers proved to be versatile under a wide range of annealing temperatures between 50 and 60°C. No other background or non-specific bands were seen on PCR analyses. The diagnostic specificity of the primers was tested using many non-standard clinical isolates not including *Enterococcus* sp., where only amplification of the gene for ribosomal RNA was seen in most cases (see below). Unfortunately, standard or clinical isolates of *Enterococcus* sp. were not available for further analysis. So far, no other species of *Enterococcus* has been reported among many microorganisms detected in the oral cavity. One previous study of 22 strains of *Enterococcus* isolated from the canals of root-filled teeth found no species other than *E. faecalis* (26). In fact, *E. faecalis* is reported to account for 80% of enterococcus infections in humans, whereas *E. faecium* accounts for the remaining 20% (16). Therefore, any false positive identification of *E. faecalis* in samples obtained from root canals seems unlikely.

As seen in Figs. 1 and 2, the smaller fragments (650 and 803 bp) correspond to the coding regions of GroES/EL chaperone protein and iron-sulfur binding protein, respectively. The specificity of the chaperone gene was previously demonstrated by testing multiple *Enterococcus* species (16). The iron-sulfur binding gene was selected randomly in this study. A BLAST search for the corresponding primers of both genes would yield promising specificity for *E. faecalis*.

The larger fragment (1522 bp) corresponding to the full-length 16S rRNA gene of *E. faecalis*, which was repeated 4 times along the genome, was shown to be semi-specific for detection of the bacterium (Fig. 2). This band was not observed after culturing microbial samples lacking *E. faecalis* in medium supplemented with 6.5% sodium chloride. Due to the conserved status of this sequence among bacterial genomes, the primers could be used to target similar regions in other bacteria. Thus, these primers could serve as an internal control for the detection of *E. faecalis* where amplification of the ~1500-bp fragment alone not only confirms the absence of *E. faecalis*, but also verifies the consistency of the entire procedure, starting with sampling and ending with gel analysis. Despite these findings, use of the 16S rRNA gene with different sets of primers has been widely employed by many workers in genotyping and diagnostic studies (27).

Some species of black-pigmented bacteria, including

Peptostreptococcus sp., *Fusobacterium* sp., *Eubacterium* sp., and *Actinomyces* sp., have been associated with certain clinical signs and symptoms (28). However, no absolute correlation has been established between any particular species and the severity of endodontic infections (22). Since *E. faecalis* is a commonly occurring component of the intestinal microflora, and an occasional pathogen of the urinary tract in women, variations in prevalence among different studies could simply indicate the influence of individual health practices and dietary habits, and hence the opportunistic nature of primary infection. In other words, *E. faecalis* in primary infections of necrotic root canals could originate from fecal contamination.

According to the data summarized in Tables 2 and 3, in terms of clinical signs and symptoms, *E. faecalis* infection was found in 4 (10.5%) of 38 asymptomatic patients and 2 (9.09%) of 22 symptomatic patients, and the difference in incidence between these groups was not significant ($P > 0.05$). This result differs from a similar previous study that noted an incidence of 11.5% in asymptomatic teeth versus 3.7% in symptomatic cases (2). The results of this study using a multiplex PCR assay indicated that *E. faecalis* is not prevalent in primary infections of necrotic root canals (10%). Furthermore, the prevalence of *E. faecalis* in cases of primary infections revealed that this species was more frequently detected in asymptomatic than in symptomatic cases (2).

The present study did not establish a significant association between clinical signs and symptoms and presence of *E. faecalis* in necrotic root canals. Further studies using a higher number of specimens may be required to reconfirm this data. Additional work will also be required to screen the specificity of primers through application to other enterococcal species. Due to the persistence of *E. faecalis* after routine disinfection procedures during root canal therapy, patients who test positive for infection are at risk of secondary infection and treatment failure. A long-term follow-up study would yield useful findings in this respect.

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