Prevalence, Phenotype, and Genotype of *Enterococcus faecalis* Isolated from Saliva and Root Canals in Patients with Persistent Apical Periodontitis

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Abstract

Introduction: The aim of this study was to investigate the prevalence, phenotype, and genotype of *Enterococcus faecalis* isolated from saliva and root canals in patients with endodontic treatment failure. **Methods:** Samples were collected from 32 adults undergoing retreatment for periapical lesions after endodontic treatment performed at least 2 years previously. Isolates that were presumed to be *E. faecalis* were identified by both API20 Strep kits and 16S rRNA sequencing. Phenotypic tests for hemolysin and gelatinase production and antibiotic susceptibility were performed. Genotype analysis comprised virulence gene detection and pulsed field gel electrophoresis (PFGE). **Results:** The prevalence of *E. faecalis* was 18.8% in saliva and 40.6% in root canals ($P = .666$, Fisher exact test). Of the 19 isolates of *E. faecalis,* 6 were from saliva and 13 were from root canals. In 3 patients, *E. faecalis* isolates from saliva were more resistant to gentamicin than those from root canals. The genes *ace*, *asa*, *gelE*, *cylA*, and *efaA* were detected from all isolates. PFGE after *Sma*I digestion showed a genetic correlation among all isolates of 62%–100%. **Conclusions:** Phenotype and genotype evidence of potential virulence factors was identified in *E. faecalis* from both saliva and root canals. A single patient might carry different *E. faecalis* strains in saliva and root canals. *(J Endod 2010;36:1950–1955)*

Key Words

Enterococcus faecalis, genotype, phenotype, prevalence, root canals, saliva

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Enterococcus faecalis bacteria are gram-positive cocci. They are common inhabitants of the human gastrointestinal and genitourinary tracts (1). In dentistry, *E. faecalis* has been associated with caries lesions (2), with chronic periodontitis (3), and very frequently with persistent apical periodontitis (4).

Although the most obvious source of *E. faecalis* in filled root canals is the oral cavity, there is little evidence to show this is the case. *E. faecalis* has not been found in the oral cavity of young infants (5) and was detected in saliva samples from only 11 of 100 patients receiving endodontic treatment and 1 of 100 dental students with no history of endodontic treatment (6). Furthermore, no published data exist on the presence of *E. faecalis* in the oral cavity of patients with persistent apical periodontitis. It is thus currently unknown whether *E. faecalis* isolated from the oral cavity differs from that isolated from filled root canals.

The role of *E. faecalis* in root canal infections also remains unclear. It had been thought that *E. faecalis* not only possesses various virulence factors but also is able to share these virulence traits among species to further contribute to its survival and ability to cause infection (7). On the other hand, its ability to survive and persist as a pathogen in root canals makes it a more important virulence factor (8). The investigation of phenotype and genotype of *E. faecalis* would be useful in understanding this bacterium's role in endodontic infections.

In this study, the prevalence and phenotypic and genotypic characteristics of *E. faecalis* isolated from saliva and root canals of patients with persistent apical periodontitis were investigated and compared.

Materials and Methods

Study Participants

Patients aged 18 years or older who attended the Hospital of Stomatology, Peking University, were recruited for this study. They had all sought root canal retreatment for periapical lesions after root canal treatment that had been performed at least 2 years previously. Patients who were pregnant, had active caries or periodontal disease (probing depth greater than 5 mm), present of a sinus, had used local or systemic antimicrobial agents during the 6 months before the study, smoked, or had diabetes or other systemic conditions were excluded. Approval for conducting the study was granted by the Peking University Institutional Review Board. Written consent to participate in the study was obtained from all participants.

Sample Preparation

Saliva samples were obtained from all participants. The sampling and processing procedures were based on the concentrated oral rinse method and were performed as previously described (6). Briefly, patients rinsed their mouths for 60 seconds with 10 mL sterile distilled water and transferred the oral rinse sample to a 50-mL polypropylene tube. Samples were immediately processed or stored at 4°C. Root canal sampling was carried out during patient treatment according to principles outlined by Pinheiro et al (9, 10). Aseptic techniques were used throughout endodontic therapy and sample acquisition. All coronal restorations, posts, and carious defects were first removed. Teeth were isolated from the oral cavity with a rubber dam and disinfected with 30%

hydrogen peroxide and then 2.5% sodium hypochlorite, which was inactivated by 5% sodium thiosulfate. The root filling was removed with Gates Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland) and endodontic files without chemical solvents. Irrigation was performed with sterile saline solution to remove any remaining materials and to moisten the canal before microbial sample collection. A sterile absorbent paper point was introduced into the full length of the canal (as determined with a preoperative radiograph), kept in place for 60 seconds, and transferred to transport medium.

Microbial Isolation and Identification

All samples were processed in the laboratory within 2 hours of collection. Each saliva sample was centrifuged at 4°C for 10 minutes at 13,000*g*. The supernatant was discarded, and the pellet was resuspended in 1 mL of sterile water. The transport medium containing the paper point from the root canal was shaken thoroughly in a mixer for 60 seconds, and a 50-μL aliquot was plated onto bile esculin azide (BEA) agar (Difco; Becton Dickinson, Sparks, MD) to selectively isolate enterococci. The aliquot was spread with a spiral plater (Model D; Spiral Systems, Inc, Cincinnati, OH), and plates were incubated aerobically for 24–48 hours at 37°C. Colonies presumptively identified as enterococci on the basis of BEA hydrolysis were streak-plated onto fresh BEA agar plates. Isolates were then characterized as gram-positive, catalase-negative, and non-motile cocci if they were capable of growth at 42°C in Todd Hewitt Broth (THB) (Difco; Becton Dickinson) agar supplemented with 6.5% NaCl. Finally, we identified *E. faecalis* by using API 20 Strep identification kits (Analytical Profile Index; Bio Mérieux SA, La Balme les, France), with *E. faecalis* ATCC 29212 strain serving as a positive control.

We performed 16S rRNA sequencing on all enterococcal strains to confirm their identity. Total DNA extractions were performed by using the TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. Total DNA was resuspended in sterile nuclease-free water, quantified by using a multichannel spectrophotometer (Beckman-Coulter DU_ 604; Foster City, CA), and adjusted to a final concentration of approximately 100 ng/μL. We performed polymerase chain reaction (PCR) by using sequence-specific primers derived from full-length *E. faecalis* 16S rRNA (Table 1), as previously described (11). Briefly, PCR amplifications were prepared in a 25-μL final reaction volume containing 1 μL total DNA template, 0.5 μL of each primer, 0.5 μL deoxyribonucleoside triphosphate (Invitrogen, Carlsbad, CA), 0.5 μL HotStarTaq DNA polymerase (Qiagen, Valencia, CA), and 2.5 μL 10X PCR buffer (Qiagen, Valencia, CA). PCR conditions were as follows: 15 minutes of initial enzyme activation/DNA denaturation at 95°C, followed by 35 cycles at 94°C for 20 seconds, 68°C for 45 seconds, and 72°C for 15 seconds. PCR products were analyzed by electrophoresis in 1.5% agarose gels containing ethidium bromide and visualized by ultraviolet fluorescence by using an AlphaImager 2200 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA).

Hemolysin and Gelatinase Production

We assessed all strains for hemolytic activity by streaking a single colony onto a plate of THB agar supplemented with 4% defibrinated horse blood or sheep blood, which was incubated at 37°C for 48 hours. Hemolysin activity was detected as a clear halo around a colony. We assessed gelatinase activity by streaking a single colony onto a plate of agar containing 3% gelatin, which was incubated at 37°C for 48 hours. Gelatinase activity was detected as a clear halo around a colony.

TABLE 1. Primers Used to Identify *E. faecalis* (11)

Gene	Sequence	Product size (bp)
Ef16SF	5′-CCGAGTGCTTGCACTCAATTGG-3′	138
Ef16SR	5′-CTCTTATGCCATGCGGCATAAAC-3′	
U16SF	5′-TTAAACTCAAAGGAATTGACGG-3′	170
U16SR	5′-CTCACGRCACGAGCTGACGAC-3′	

bp, base pairs.

Antibiotic Susceptibility

We determined minimal inhibitory concentrations (MICs) by using the E-test (Biodisk AB, Solna, Sweden) (12), following the procedures recommended by the manufacturer. A 0.1-mL aliquot of a standard bacterial suspension was adjusted to an optical density of 0.5 at 605 nm and spread evenly over the surface of a plate of Mueller-Hinton agar (Difco; Becton Dickinson). After we applied E-test strips, plates were incubated aerobically at 35°C for 16–20 hours, and MICs were read from the interpretive scale at the point at which the ellipse of inhibition intersected the strip. The following antibiotics were tested: penicillin, ampicillin, chloramphenicol, erythromycin, metronidazole, tetracycline, vancomycin, gentamicin, and streptomycin. The breakpoints used for interpretation were recommended by the Clinical and Laboratory Standards Institute update to the sixteenth informational supplement.

Virulence Gene Detection

Primers that targeted segments of enterococcal virulence determinants have been previously described (11, 13, 14). The determinants tested were aggregation substance (*asa* and *asa373*), surface adhesin (*esp*), cytolysin activator (*cylA*), gelatinase (*gelE*), endocarditis antigen (*efaA*), and collagen binding antigen (*ace*) (Table 2). The PCR conditions were modified to accommodate a common annealing temperature of 56°C on the basis of initial temperature gradient profiles for each PCR target. A 1-μL sample of each total DNA template was mixed with 0.5 μL of each respective primer, 0.5 μL deoxyribonucleoside triphosphate, 0.5 μL HotStarTaq DNA polymerase, and 2.5 μL 10X PCR buffer. The PCR conditions were as follows: 15 minutes of initial enzyme activation/DNA denaturation at 95°C, followed by 35 cycles at 94°C for 20 seconds, 56°C for 45 seconds, and 72°C for 30 seconds. PCR products were separated by electrophoresis by using 1.5% agarose gels containing ethidium bromide and analyzed under ultraviolet light. All PCR products were purified and sequenced in SinoGenoMax Co, Ltd. We compared each nucleotide sequence with the NCBI GenBank database for final verification.

Genetic Correlation Analysis

Genetic profiles of total cellular DNA were obtained by pulsed field gel electrophoresis (PFGE), which was conducted at the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, as previously described (15). First, digestion was performed with 30 U of *Sma*I (New England Biolabs, Hitchin, UK) for 2 hours at 30°C. Genomic DNA restriction fragments were separated on 0.8% agarose gels, which were exposed to contour homogeneous electric fields (CHEF-DR III; Bio-Rad, Hercules, CA). The pulse time was increased from 5 to 35 seconds during a period of 30 hours at 6 V/cm (16). Gels were stained with ethidium bromide, destained in distilled water, and photographed under ultraviolet illumination.

Results

Prevalence of *E. faecalis*

A total of 19 isolates of *E. faecalis* were obtained among the 32 retreatment cases, 6 from saliva samples and 13 from root canal

TABLE 2. Primers Used to Detect Virulence Genes (11, 13, 14)

Gene	Sequence	Product size (bp)
esp	espF 5′-TTGCTAATGCTAGTCCACGACC-3′ espR 5′-GCGTCAACACTTGCATTGCCGAA-3′	933
efaA	efaAF 5′-GCCAATTGGGACAGACCCTC-3′ efaAR 5′-CGCCTTCTGTTCCTTCTTTGGC-3′	688
ace	aceF 5′-GGAATGACCGAGAACGATGGC-3′ aceR 5′-GCTTGATGTTGGCCTGCTTCCG-3′	616
cylA	cylAF 5′-GACTCGGGGATTGATAGGC-3′ cylAR 5′-GCTGCTAAAGCTGCGCTTAC-3′	688
gelE	gelEF 5′-ACCCCGTATCATTGGTTT-3′ gelER 5′-ACGCATTGCTTTTCCATC-3′	405
asa	asaF 5′-CCAGCCAACTATGGCGGAATC-3′ asaR 5′-CCTGTCGCAAGATCGACTGTA-3′	529
asa373	asa373F 5′-GGACGCACGTACACAAAGCTAC-3′ asa373R 5′-CTGGGTGTGATTCCGCTGTTA-3′	619

bp, base pairs.

samples. The prevalence of *E. faecalis* was thus 18.8% (6/32) in the saliva and 40.6% (13/32) in the root canals ($P = .666$, Fisher exact test). API20 Strep kits and 16S rRNA sequencing got the same results identifying *E. faecalis* in this study.

Phenotype of *E. faecalis*

No hemolysin or gelatinase activity was detected among the 19 isolates, and all were sensitive to penicillin, ampicillin, and vancomycin but resistant to metronidazole and streptomycin. *E. faecalis* was detected in both root canal and oral rinse samples in patient numbers 3, 33, and 37. No obvious differences were found in phenotype between these 3 "pairs" of isolates, except that *E. faecalis* bacteria from the saliva samples were more resistant to gentamicin than those from the root canal samples.

Virulence Genes

Genes *ace, asa, cy1A, efaA,* and *gelE* were detected in all isolates tested, including the reference strain ATCC 29212. The *esp* gene was present in 9 (47.4%) of 19 isolates tested, 4 from saliva samples and 5 from root canal samples. The *asa373* gene was not detected in any strain (Table 3).

Genetic Correlation

Digestion of 21 *E. faecalis* isolates (including ATCC 29212 and ATCC 51299 reference strains) with *Sma*I followed by PFGE showed that genetic correlation of all isolates ranged from 62%–100% (Fig. 1). Fifteen isolates showed a similar profile to ATCC 29212, even though they were obtained from different patients. In contrast, strains from different sources (saliva versus root canals) in the same patients (P33.S and P33.RC, P37.S and P37.RC) had different PFGE profiles.

Discussion

No significant differences were found on the *E. faecalis* prevalence in this study between saliva samples and root canal samples. The root canal system and the oral cavity are closely connected environments especially in the presence of coronal leakage. Thus, it is feasible that *E. faecalis* species in the root canal originates from the oral cavity or that species in the root canal spreads to the oral cavity. The exact origin of these *E. faecalis* organisms, however, remains unknown. Because of the ubiquitous presence of *E. faecalis*, root canal infections might arise from different sources (17).

TABLE 3. Genotypic Characteristics of *E. faecalis* Isolated from Filled Root Canals and Saliva Samples

Strain	*ace*	*asa*	*cy1A*	*efaA*	*gelE*	*asa373*	*esp*
P. 1 R.C.	+	+	+	+	+	—	—
P. 3 S.	+	+	+	+	+	—	—
P. 3 R.C.	+	+	+	+	+	—	—
P. 5 R.C.	+	+	+	+	+	—	—
P. 21 R.C.	+	+	+	+	+	—	—
P. 23 R.C.	+	+	+	+	+	—	—
P. 24 R.C.	+	+	+	+	+	—	—
P. 25 R.C.	+	+	+	+	+	—	—
P. 31 R.C.	+	+	+	+	+	—	+
P. 33 S.	+	+	+	+	+	—	+
P. 33 R.C.	+	+	+	+	+	—	—
P. 35 S.	+	+	+	+	+	—	+
P. 37 S.	+	+	+	+	+	—	+
P. 37 R.C.	+	+	+	+	+	—	+
P. 39 R.C.	+	+	+	+	+	—	+
P. 40 R.C.	+	+	+	+	+	—	+
P. 52 S.	+	+	+	+	+	—	+
P. 57 S.	+	+	+	+	+	—	—
P. 58 R.C.	+	+	+	+	+	—	+
ATCC 29212	+	+	+	+	+	—	—

R.C., root canal, S., saliva.

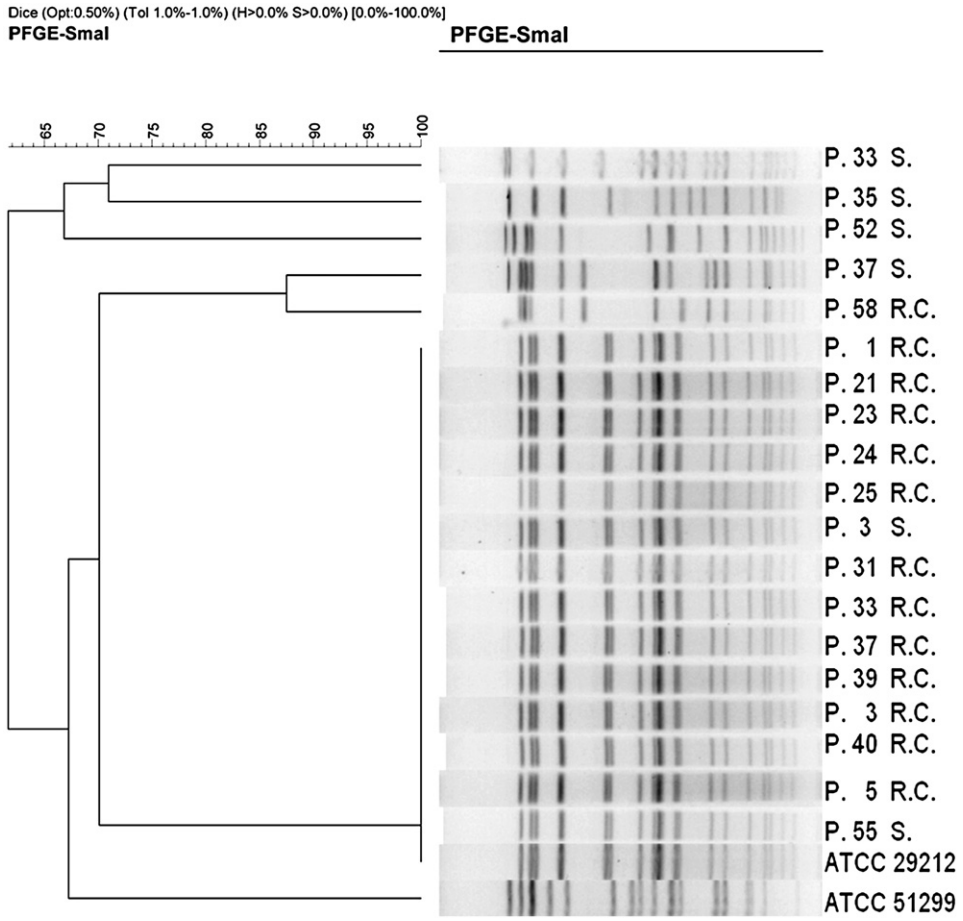

Figure 1. Dendrograms showing similarity of 21 *E. faecalis* strains. PFGE of SmaI restriction fragments from *E. faecalis* strains isolated from root canals and saliva.

Previous studies investigating *E. faecalis* in root-filled teeth with periradicular lesions have demonstrated a prevalence ranging from 24%–77% (4). This wide range might be attributable to different identification techniques, geographic differences, or sample sizes of different studies (18). The authors recognize the limitation of the cross-sectional studies; they capture snapshots of microflora existing in a complex dynamic environment and are not reproducible even within the same individual on different occasions. Future studies could longitudinally examine the prevalence and characteristics of enterococci recovered from saliva and root canals. Different sampling methods might also lead to different results. The sampling method in present study might be effective for the planktonic bacteria and bacteria from the biofilm on root canal filling but not for the bacteria from biofilm on oral mucosa or root canal dentin.

Commercial bacteria-identification kits are widely used in epidemiologic investigations, including the identification of enterococcal species from diverse sources of human infections such as the oral cavity (6, 11) and root canals (9, 19). However, research has shown discordances between the results of commercial tests and those from conventional molecular identification methods, especially in cases of atypical enterococcal strains (20). Therefore, in this study, the results from tests with biochemical identification kits (API20 Strep kits) were compared with those from 16S rRNA sequencing. No difference was found between these 2 methods in this study.

Although the *gelE* gene was found in all isolates, none of them expressed gelatinase, which hydrolyzes gelatin, casein, insulin, fibrinogen, and small peptides (21). Because the secretion of gelatinase can be auto-regulated through the "fsr" system, the detection of the *gelE* gene by PCR does not correlate with its phenotypic expression, and "silent" genes, such as *fsr*, might control the expression of gelatinase (22). The expression of gelatinase might be related to caries status. In 1975, Gold et al (23) described *E. faecalis* 2 SaR (now known as OG1) as a strain recovered from a human oral cavity that exhibited gelatinase activity and was capable of inducing caries in germ-free rats. Despite no *gelE* gene records, only 36.4% (4/11) of strains from saliva samples were previously reported to produce gelatinase (6); 3 of these 4 isolates came from patients with active caries. In another study (11), 8 of 31 *gelE* positive isolates expressed no gelatinase; caries status was unavailable. Selecting participants without active caries might have contributed to the low expression of gelatinase in this study. Further studies could examine the relationship between caries status and gelatinase production by oral *E. faecalis*.

Regarding treatment of endodontic infections, the lack of circulation in root canals makes it inefficient to use systemic antibiotics; hence, topical antimicrobial therapies and mechanical instrumentation are necessary (24). In addition, the conditions affecting the presence and growth of bacteria in filled root canals differ from those in primary infections. The lack of sufficient nutrition and the presence of antimicrobial agents might reduce or eliminate sensitive strains, whereas more resistant species such as *E. faecalis* might survive. The existence of multiresistant *E. faecalis* strains has been reported (1), including strains that are resistant to vancomycin, which will create treatment

problems owing to the lack of therapeutic alternatives. Although all of the examined strains in this study were susceptible to vancomycin and no multiresistant enterococcal strains were isolated, these findings do not exclude the possibility that multiresistant strains might occur in endodontic infections. Of the 3 "pairs" of *E. faecalis* from the same patients, isolates from saliva samples were more resistant to gentamicin than those from root canals. Resistance to gentamicin is believed to be related to the expression of aminoglycoside-modifying enzymes (25), which can be shared among species. In obturated root canals, there is a limited variety of often 1–2 strains in total (26). In the more "open" and complicated environment of the oral cavity, *E. faecalis* is more likely to gain virulence factors (such as aminoglycoside-modifying enzymes) from other species, thereby extending its survival and increasing its ability to cause disease (7).

All strains in this study possessed genes for the adherence factors EfaA and Ace; the latter might aid the attachment of *E. faecalis* to dentin (27). The gene related to hemolysin, *cylA*, was also found in all strains. The *esp* gene, which is associated with colonization and persistence of *E. faecalis* in urinary tract infections (28), was found in 47.4% of all isolates. Another adherence factor, aggregation substance, is a pheromone-inducible surface protein whose expression can increase adhesion to collagen type I, but not type IV (29). A PCR product representing the aggregation substance gene *asa* was found in all strains, but the gene for another type of aggregation substance, pAM373, which bears little homology, was not evident in the clinical isolates.

Finally, PFGE has been useful in the molecular epidemiologic typing of enterococci (30) and subtyping of *E. faecalis* (31), because DNA fragments from digestion by rare restriction enzymes are too large to be resolved by conventional agarose gel electrophoresis. In this study, PFGE of DNA from endodontic *E. faecalis* isolates demonstrated genotypic polymorphism, but with evidence of several clonal groups, that is, genetically related isolates (32). Genetic correlation, ranging from 62%–100%, suggests the existence of groups of genetically similar strains from different patients. *E. faecalis* is able to colonize diverse niches because of their exceptional capacity to grow in different environments, such as soil, surface waters, as well as in vegetables and olives (33). Thus it is quite possible that different people living in the same city get genetically similar strains in their saliva. The use of an additional restriction enzyme with a different recognition sequence could provide more information about the genome (34). Fifteen isolates showed a similar profile to ATCC 29212, even though they were obtained from different patients. Interestingly, P33 seemed to carry 2 different *E. faecalis* strains. The *esp* gene detection also showed a different result about the 2 strains from P33. The presence of different strains of *E. faecalis* in the same patient might suggest that a single individual can harbor different *E. faecalis* strains in different niches of the oral cavity (34). Furthermore, saliva is a more open and complicated environment; a microorganism in saliva changes at all times. Different *E. faecalis* strains can enter saliva at any time and be detected. The strains in root canals seemed less possible to change.

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