# Diversity of *Enterococcus faecalis* Genotypes from Multiple Oral Sites Associated with Endodontic Failure Using Repetitive Sequence-based Polymerase Chain Reaction and Arbitrarily Primed Polymerase Chain Reaction

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## Abstract

**Introduction:** The aim of this study was to evaluate the diversity and similarity of *Enterococcus faecalis* genotype isolates from multiple oral sites using repetitive sequence-based polymerase chain reaction and arbitrarily primed polymerase chain reaction (AP-PCR). **Methods:** Forty-two endodontically treated teeth with apical periodontitis were selected. A total of 126 microbial samples were collected from 3 different sites (saliva, pulp chamber, and root canals, all $n$ = 42) during the nonsurgical retreatment procedures. After growth on m-Enterococcus agar, the colonies were isolated, characterized as gram-positive catalase negative cocci, and identified using an API 20 Strep kit (bioMérieux, Marcy-l'Etoile, France). Seventy-four colonies from 10 patients were confirmed as *E. faecalis* by polymerase chain reaction (16S ribosomal RNA). Repetitive sequence-based polymerase chain reactions using ERIC and AP-PCR using RW3A primers were performed in all 74 colonies. Fingerprints were analyzed and separated into genotypic groups based on the Dice coefficient percentage of similarity (82% or greater) as determined by ERIC reproducibility assays involving *E. faecalis* controls. **Results:** Seven different *E. faecalis* genotypes (GTs) (GT1 = 27%, GT2 = 17.6%, GT3 = 1.3%, GT4 = 18.9%, GT5 = 9.5%, GT6 = 14.9%, and GT7 = 10.8%) were observed in different subjects and oral sites associated with endodontic failure. Remarkably, in 4 of 5 patients, the same GTs present in the infected root canals were also isolated from either the pulp chamber or the saliva samples. In particular, GT6 was detected in all 3 oral sites of patient 37. **Conclusions:** *E. faecalis* GTs isolated from saliva, the pulp chamber, and the root canal were similar using the Rep-PCR and AP-PCR methods. These findings suggest that coronal microleakage is a conceivable cause of endodontic failure. *(J Endod 2016;■:1–6)*

## Key Words

Arbitrarily primed polymerase chain reaction, endodontics, *Enterococcus faecalis*, microleakage, microorganisms, repetitive sequence-based polymerase chain reaction, saliva

**Significance**

This study showed that the multiple *E. faecalis* genotypes present in the saliva or pulp chamber may be the same isolated from root-filled teeth with apical periodontitis. These findings suggest that coronal microleakage may be a potential cause of endodontic failure.

Conventional endodontic therapy usually fails when the treatment is performed inadequately (1). However, there are some instances in which procedures follow the highest standards and still result in nonhealing of apical periodontitis (2).

There is enough evidence in the literature that endodontic failure may take place because of the ability of some microorganisms to survive after current treatment protocols (3, 4). Also, it has been shown that these microorganisms can gain access to the root canal system either during or after treatment because of coronal microleakage (5, 6).

*Enterococcus faecalis* is a nonmotile, gram-positive, spherical bacterium. It can be observed singly, in pairs, or in short chains and is most often found in the large intestine of humans. It is a facultative anaerobe with a fermentative metabolism (7).

*E. faecalis* is listed as the first to the third leading cause of nosocomial infections. Most of these infections occur after surgery of the abdomen or a puncturing trauma but can also be linked to the increased use of intravenous lines and catheters, which are considered compromising devices. It is also responsible for urinary tract infections,

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bacteremia, endocarditis, meningitis, and foodborne disease. It can be found in wound infections and intra-abdominal abscesses, along with many other bacteria (8).

*E. faecalis* has been frequently recovered from the root canals of teeth with apical periodontitis (9, 10) and is implicated as the major endodontic pathogen in secondary infections (4, 11–17). The epidemiology of enterococcal infections is an important part of dealing with these multiresistant organisms.

Polymerase chain reaction (PCR)-based DNA fingerprinting of microorganisms has been developed using a wide variety of techniques and primer designs. The basis of PCR-based DNA fingerprinting is that the primers can bind to specific regions of the DNA, and when this binding occurs in the proper orientation and within an optimum distance, species- or strain-specific amplification products may be generated. Primers such as REP1R and REP2, which are derived from the repetitive extragenic palindromic sequences found primarily in gram-negative bacteria (18), have been used in the technique known as repetitive sequence-based PCR (Rep-PCR) (18, 19) for studying DNA fingerprints of many bacterial species.

Arbitrarily primed PCR (AP-PCR) is any form of PCR that uses primers of arbitrary sequence and that amplifies random but discrete sequences of chromosomal DNA. AP-PCR has been used for typing bacteria. PCR is initially performed under low stringency, and the primers bind at various sites to each strand of heat-denatured chromosomal DNA; the binding of primers occurs at "best-fit" sequences and may include mismatches (20).

Because persistent root canal infection has been suggested as 1 of the factors responsible for failure, particularly in well-treated teeth, the literature has investigated the most likely sources of coronal leakage by detection of *Enterococcus* spp. Although unsatisfactory coronal restorations are associated with lower rates of complete apical healing (21), the biological aspects of coronal microleakage have not been clearly investigated. It is critical to evaluate this species occurrence in the patient's saliva and under the restorative materials compared with the root canal samples of previously endodontically treated teeth.

The literature has indicated that the overall success rate of nonsurgical retreatment of teeth with apical periodontitis is 76.7% (22). This poor prognosis may be associated with difficulties in the elimination of the particular resistant microbiota, particularly *E. faecalis*.

It would be of interest to know *in vivo* the pathways of the root canal reinfection, starting from the saliva and then entering the internal surface of the pulp chamber and spreading through the root-filled canal system. Therefore, the goals of this study were

1. To identify and locate *E. faecalis* in each clinical case using *in vivo* sampling protocols and culture techniques;
2. To analyze DNA fingerprints from the saliva, pulp chambers, and root canals of endodontically treated teeth with apical periodontitis using 2 molecular methods; and
3. To determine the diversity and similarity of genotypes present within the oral cavity.

The tracking using their genotypes may prove by DNA fingerprinting that saliva could really be a potential source of *E. faecalis* that could invade the root canal via coronal microleakage.

## Materials and Methods

### Clinical Examination

All the protocols and the specimen collection methods for this investigation were approved by the institutional review board of the Piracicaba Dental School, State University of Campinas, Piracicaba, São Paulo, Brazil. Informed consent forms were provided and signed by the patients involved in the study. Forty-two teeth of 20 patients were included. The patients were screened and scheduled to receive nonsurgical endodontic retreatment because of radiographic evidence of apical periodontitis. Medical history and dental records were obtained.

The inclusion criteria used to select teeth and patients were as follows:

1. Previously endodontically treated teeth with radiographic evidence of apical periodontitis
2. Previously endodontically treated teeth with persistence of symptoms
3. No evidence of longitudinal fractures
4. No antibiotic therapy provided 3 months before the consultation visits
5. Absence of systemic disease and periodontal disease

### Sampling Procedure

Microbial sampling of the saliva followed by the pulp chamber and the root canals was collected during the first dental appointment. Aseptic techniques were used throughout the nonsurgical root canal retreatment and before sample collection. The sampling protocols used in this study were previously detailed by Gomes et al (4, 23) and were adapted for this study as described.

### Saliva Sampling

Patients were informed to not brush their teeth or eat anything 2 hours before the appointment. One milliliter of whole saliva was collected from the patients and placed in a sterile plastic receptacle in the beginning of each section (24). Forty-two saliva samples were collected from 20 patients. The number of saliva samples was related to the number of teeth treated per patient, even if their saliva samples had already been previously collected.

### Pulp Chamber Sampling

Rubber dam isolation was placed in all teeth. The external surfaces of the crowns were then disinfected with 30% hydrogen peroxide followed by 2.5% sodium hypochlorite. These solutions were inactivated with 5% sodium thiosulfate in order to avoid interference with bacteriological sampling. Afterward, the internal surfaces of the coronal restorations or posts were collected using a sterile swab.

### Root Canal Sampling

The access cavities were disinfected using the same protocol as described previously. The root canal filling materials were then removed with Gates-Glidden drills and K-files (Dentsply Maillefer, Ballaigues, Switzerland) without the use of endodontic solvents. Sampling was performed during all retreatment procedures. Not only the gutta-percha but also debris was collected. All the canals were kept moist with sterile saline solution before the final sampling was taken with sterile paper points. The paper points were placed to the working length of the canals determined by an electronic apex locator (Novapex; Forum Engineering Technologies, Rishon Lezion, Israel). The sterile paper points were maintained inside the root canals for 60 seconds in order to absorb as much fluid as possible.

A total of 126 microbial samples were collected, 42 from each of the 3 sites (ie, the saliva, pulp chamber, and root canal of previously treated teeth), during the retreatment procedures. The microbial evaluation of the single-canal ecological environment was evaluated. The root with radiographic evidence of apical periodontitis was selected in teeth with multiple canals. In teeth in which all the roots showed periapical lesions, the largest canals were then chosen.

## Culture Procedure

All samples were transferred immediately to the Viability Medium Götemborg Agar (VMGA III) transport medium (25) and promptly taken to the microbiology laboratory for processing in a maximum period of 4 hours. Inside the chamber, each transport medium was shaken thoroughly in a mixer for 60 seconds (Vortex; Marconi, São Paulo, SP, Brazil). The clinical samples were plated using sterile plastic spreaders onto m-Enterococcus agar (Difco Laboratories, Becton, Dickinson and Company, Sparks, MD) and incubated at 35°C for 2 days in duplicate plates. Based on colony morphology, presumptive *E. faecalis* colonies were subcultured to obtain purity, Gram stained, tested for catalase activity, and identified using an API 20 Strep kit (bioMérieux, Marcy-l'Etoile, France).

## DNA Fingerprinting

The API identifications of strains from saliva ($n$ = 10), pulp chambers ($n$ = 17), and root-filled canals ($n$ = 47) of 10 clinical cases from 8 patients were confirmed via PCR with *E. faecalis* 16S ribosomal RNA probes Ef16SF CCG AGT GCT TGC ACT CAA TTG G and Ef16SR CTCTTATGCCATGCGGCATAAAC as described by Sedgley et al (26).

*E. faecalis* clinical isolates extracted with a MasterPure kit (Epicentre Technologies, Madison, WI) and DNA fingerprints of 74 strains from saliva ($n$ = 10), pulp chambers ($n$ = 17), and root-filled canals ($n$ = 47) of 10 clinical cases were developed using 2 different standard fingerprinting methods: Rep PCR (primers ERIC1 and ERIC2) and AP-PCR (primer RW3A). The amplifications were performed as soon as possible after preparation of the master mixture. Both were performed in an ultraviolet sterile biohood by modification of published protocols (18).

Rep-PCR master mixture per reaction consisted of 100 pmol of each ERIC primer, 2.5 mmol/L $MgCl_2$, 1.75 U AmpliTaq Gold Polymerase (Applied Biosystems, Foster City, CA), 0.2 mmol/L deoxynucleotide triphosphates, 5 $\mu$L 10× AmpliTaq Gold (Applied Biosystems) buffer, and 200 ng DNA in a 50-$\mu$L reaction volume. The amplification was performed in a thermocycler (PTC-200; MJ Research, Waltham, MA) with an initial denaturation of 95°C for 7 minutes followed by 30 cycles of denaturation at 90°C for 30 seconds, annealing at 52°C for 1 minute, and extension at 65°C for 8 minutes. A final extension was performed at 65°C for 16 minutes.

AP-PCR master mixture per reaction consisted of 100 pmol RW3A primer, 2.5 mmol/L $MgCl_2$, 1.75 U AmpliTaq Gold Polymerase, 0.2 mmol/L deoxynucleotide triphosphates, 5 $\mu$L 10× Amplitaq Gold buffer, and 200 ng DNA in a 50-$\mu$L reaction volume. The amplification was performed in a thermocycler (PTC-200) with an initial denaturation of 95°C for 7 minutes followed by 30 cycles of denaturation at 90°C for 30 seconds, annealing at 52°C for 1 minute, and extension at 65°C for 8 minutes. A final extension was performed at 65°C for 16 minutes.

The PCR products were separated by gel electrophoresis with a 1.5% agarose gel and Tris-acetate–EDTA running buffer. The gels were stained with ethidium bromide and then photographed with an ultraviolet light source and visualized using an ultraviolet transilluminator imaging system (MiniBIS Pro; DNR Bio-Imaging Systems, Jerusalem, Israel).

Genomic fingerprints of the *E. faecalis* isolates were analyzed and separated into genotypes based on the Dice coefficient percentage of similarity (82% or greater) as determined by ERIC reproducibility assays involving *E. faecalis* controls. Gel Compar II software (Applied Maths, Kortrigk, Belgium) was used to determine clonal types and generate similarity dendrograms based on the Dice coefficient and the unweighted pair group method with arithmetic mean dendrogram type.

## Statistical Analysis

The data collected from each case were entered into a spreadsheet and analyzed statistically using SPSS for Windows (SPSS Inc, Chicago, IL).

## Results

*E. faecalis* was isolated from 10 clinical cases involving 8 patients. Seventy-four *E. faecalis strains* were biochemically identified; 10 were isolated from the saliva, 17 from the pulp chamber, and 47 from the root canal. The API identifications of strains were confirmed via PCR with *E. faecalis* 16S ribosomal RNA probes; all of them were identified as *E faecalis*. DNA fingerprints of 74 strains using 2 different standard fingerprinting methods were performed: Rep-PCR (primers ERIC1 and ERIC2) and AP-PCR (primer RW3A) showed different results.

According to criterion adopted in the present study, isolates showing patterns with differences starting on 2 (ERIC primer) or 3 (RW3A primer) bands were considered different. Seven genotypes groups were identified among the 74 isolates. Differences in band intensity at matching positions among some isolates made visual interpretation somewhat difficult although all were classified as clonally related by PCR tested methods after repeated experiments. By comparing techniques, most isolates classified as identical, related, or different by Rep-PCR with the ERIC primers were also classified in the same manner by AP-PCR (Table 1 and Fig. 1).

Monoclonal colonization of *E. faecalis* was observed in infected root-filled canals. Homogeneous *E. faecalis* genotypes were observed in different subjects and particularly in multiple colonies isolated from each sampling site. Seven different genotypes (GT1–GT7) were identified among the 74 *E. faecalis* isolates from the 3 sites investigated as follows: GT1 = 27%, GT2 = 17.6%, GT3 = 1.3%, GT4 = 18.9%, GT5 = 9.5%, GT6 = 14.9%, and GT7 = 10.8%. In all 10 cases, 6 out of the 7 clonal types were present in the infected root-filled canals, except genotype C, which was found only in the saliva site. Notably, in 4 out of the 8 patients, the same *E. faecalis* clonal type, which was present in the root canal infection, was also isolated from either the pulp chamber or the saliva samples. In particular, clonal type E was detected in all 3 oral sites of patient 37 (Table 2).

## Discussion

Although similarity in the bacterial species isolated from root canals of different teeth in the same patient has already been shown using culture and PCR (27, 28), this study isolated multiple *E. faecalis* colonies from different sampling sites (10 from the saliva, 17 from the pulp chambers, and 47 from the root canals), showing homogeneous genotypes using AP-PCR and Rep-PCR. Four patients showed the same *E. faecalis* clonal type in the root canal and pulp chamber, all genetically heterogeneous. One patient presented the same clonal type in the 3 sites investigated.

DNA fingerprinting of bacteria has been extensively investigated in the last few years, typically by comparing banding patterns obtained from genomic DNA of the organisms by visual inspection without complicated mathematical calculation (19). However, there are no

**TABLE 1.** Comparison of Primes RW3A and ERIC Results for *Enterococcus faecalis* Isolates

| Clonal types | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| ERIC | CT1 | CT2 | CT3 | CT4 | CT5 | CT6 | CT7 |
| RW3A | CT6 | CT1 | CT7 | CT2 | CT4 | CT5 | CT3 |
| Number of isolates $N$ = 74 | $n$ = 20 | $n$ = 13 | $n$ = 1 | $n$ = 14 | $n$ = 7 | $n$ = 11 | $n$ = 8 |
| Frequency 100% | 27% | 17.6% | 1.3% | 18.9% | 9.5% | 14.9% | 10.8% |

A, B, C, D, E, F, and G indicate 7 different genotypes for both polymerase chain reaction methods used and are therefore organized into clonal types with different numbers.

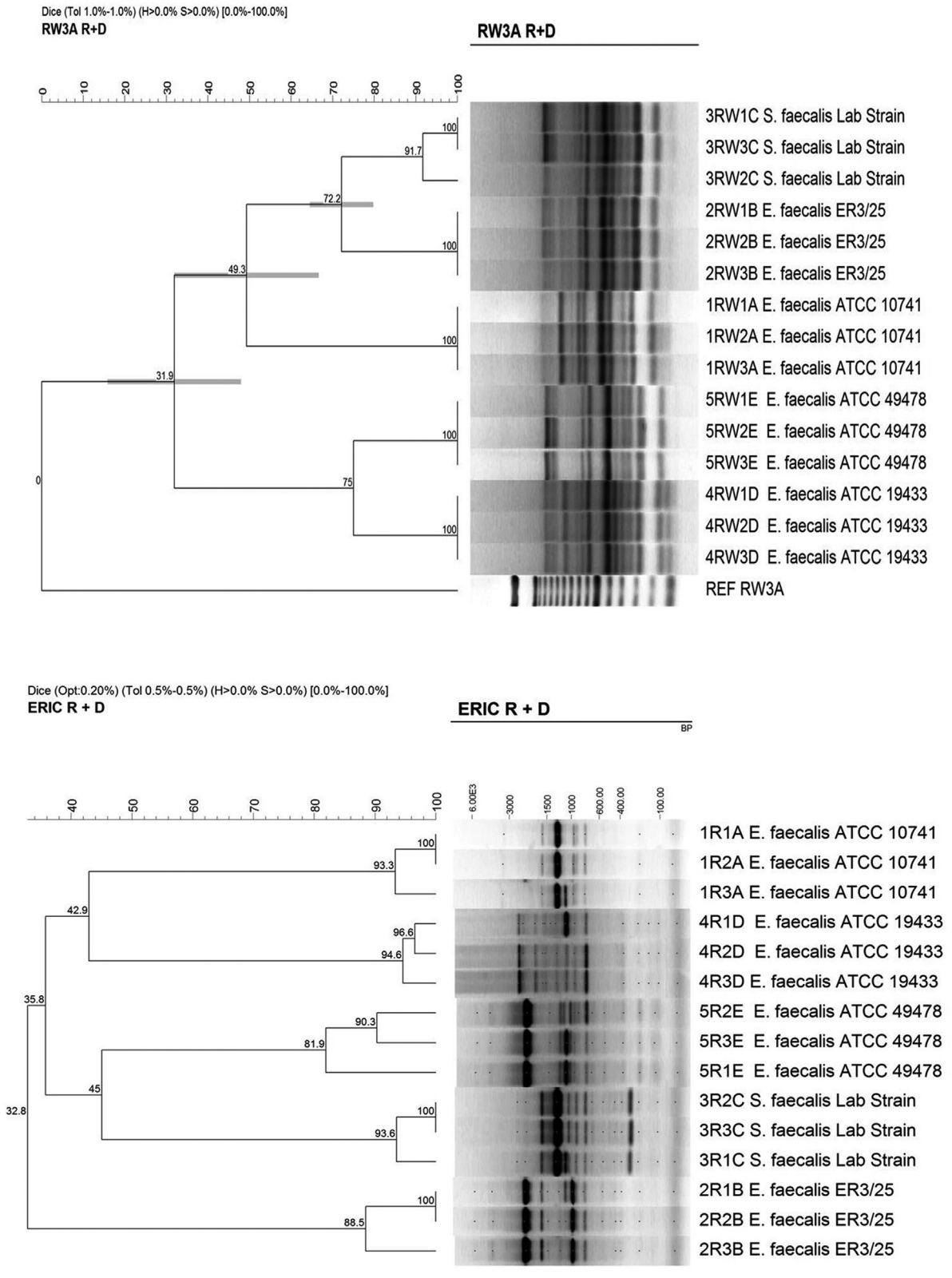


**Figure 1.** Dendrograms generated by Gel Compar II software showing percent similarity of the various isolates. Strains were designated as the same genotype if they were ≥70% similarity.

consensus methods or criteria for interpretation of the banding patterns obtained from the reactions. In the present study, isolates showing patterns with differences starting on 2 (ERIC primer) or 3 (RW3A primer) bands were considered different. On the other hand, in the study by Woods et al (19), isolates were considered to belong to the same group if their PCR patterns differed by no more than 2 bands.

**TABLE 2.** *Enterococcus faecalis* Isolates from 10 Clinical Cases but 8 Patients ($N$ = 74)

| Cases | Root canal | Pulp chamber | Saliva |
|---|---|---|---|
| # 8 | B ($n$ = 3) | B ($n$ = 1) | — |
| # 21 | B ($n$ = 9) | — | — |
| # 28 | D ($n$ = 2) | — | — |
| # 31 | A ($n$ = 10) | A ($n$ = 8) | D ($n$ = 8) |
| # 34 | G ($n$ = 8) | — | — |
| # 35 | A ($n$ = 2) | D ($n$ = 1) | — |
| # 37 | E ($n$ = 2) | E ($n$ = 4) | E ($n$ = 1) |
| # 36 | — | — | C ($n$ = 1) |
| # 40 | F ($n$ = 8) | F ($n$ = 3) | — |
| # 41 | D ($n$ = 3) | — | — |
| Total isolates | $n$ = 47 | $n$ = 17 | $n$ = 10 |

—, not applicable.

A, B, C, D, E, F, and G indicate bacterial genotypes. Cases 21/28 and 31/35 belong to the same patients ($n$ = 2).

The interpretation of Rep-PCR results was similar to those obtained by AP-PCR in identifying clonal relationships among *E. faecalis* isolates. This might be related to the lower number of bands for comparison obtained by Rep-PCR than AP-PCR and the fact that some of these bands were common to all isolates. Further studies involving DNA sequencing of these bands might prove informative.

Despite the homogeneity found in the isolates from saliva, pulp chamber, and root canal, genetic heterogeneity was observed when compared among different subjects, corroborating the results of previous studies using pulsed-field gel electrophoresis and sequence-based typing methods (28). Our research also found homogeneous genotypes into 2 different root-filled teeth of the same subject, which agrees with the findings of Pinheiro et al (28).

The occurrence of *E. faecalis* in infected root-filled canals indicates that its incidence in the oral cavity may be higher than previously suggested (5, 29–31). In our study, 4 cases of the same genotype found in the root canals were also found in the pulp chamber or in the saliva samples. It is important to stress that the external surfaces of the prosthetic crowns or restorative materials were disinfected to avoid false-positive results.

The recovery of *E. faecalis* from previously treated root canals with apical periodontitis (3, 9, 12, 13, 17, 28, 32–35) is still the subject of research. There is strong uncertainty if those persistent bacteria found in root-filled canals remained from the original infection, being able to resist the endodontic disinfection protocols, or if they came from the saliva and were able to invade the root canal system through defective restorative material, remaining there because of its specific environment, featuring a secondary infection (36). Some research indicates that *E. faecalis*, mostly present in the human intestine, is not a permanent colonizer of the oral cavity (37, 38). Consequently, the question arises of how *E. faecalis* participates in root canal infections. Another hypothesis is that *E. faecalis* transiently colonizes the oral cavity via foodborne infection (39), which is an exogenous route of infection (40). The findings of the current study could apparently support this by showing that *E. faecalis* was more frequently found in the root canal than in the saliva of the same patients.

However, the fact that *E. faecalis* was not found in most of the saliva samples does not exclude the possibility of this microorganism from this site being responsible for root filling reinfection via coronal microleakage. It takes time for microorganisms to invade the root canal and provoke a periapical reaction. The salivary microbiome, although stable (41), can suffer from the influence of human genetic makeup, diet, age, surroundings, smoking, and personal oral hygiene (42). Furthermore, the host's systemic condition (eg, obesity) is reportedly associated with the microbiota structure in saliva (41). Therefore, microorganisms from saliva are present in higher or lower quantities depending on several factors, which will differ from subject to subject.

Finding the same microorganism in different sites using the culture technique could be only a coincidence, but molecular methods used in this work showed the similarity of the strains. Additionally, multiple colonies isolated from each sampling site showed homogeneous genotypes. Root-filled canal infections appear to be attributed to a large number of different *E. faecalis* genotypes that share a large degree of homology. Whether these unique genotypes differ in terms of virulence or antimicrobial activity is not well-known and is the subject of our ongoing studies.

In conclusion, *E. faecalis* genotypes isolated from saliva, pulp chamber, and root canal were similar using Rep-PCR and AP-PCR methods. These findings suggest that coronal microleakage is a conceivable cause of endodontic failure.

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