UMSHA Press

AJDR
Avicenna Journal of Dental Research

Avicenna J Dent Res. 2025;17(2):x-x. doi:10.34172/ajdr.2147

http://ajdr.umsha.ac.ir

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Enterococcus faecalis in Untreated Root Canal Teeth: A Microbiological Study

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Article history:
Received: Xx xx, 2023
Accepted: Xx xx, 2023
ePublished: Xx xx, 2025

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Abstract

Background: Some missteps during procedures, such as insufficient disinfectant use for root canal disinfection or instrumentation, can fail the endodontic treatment. *Enterococcus faecalis* can adhere to endodontic cements and root canal walls, forming a resistant biofilm. Thus, it is often isolated from root canals in secondary/persistent infection. This study focused on detecting the presence of *E. faecalis* in teeth with untreated root canals using molecular assay.

Methods: In this study, 32 samples of untreated root canals were collected from the Dentistry Clinic of the School of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, Iran. The existence of *E. faecalis* in these samples was detected using multiple methods, such as a polymerase chain reaction (PCR) assay based on the *16SrRNA* gene and traditional culture. A disk diffusion test (Kirby-Bauer susceptibility test) was used to determine the antimicrobial susceptibility of isolated strains.

Results: *E. faecalis* was detected in six samples (18.75%) using the culture method, but four (66.67%) were confirmed by the PCR method. All *E. faecalis* isolates were sensitive to vancomycin and showed the highest resistance to ampicillin (100%) and chloramphenicol (83%), respectively.

Conclusion: Further research is needed to develop more efficient antimicrobial agents for endodontic treatment. In addition to finding more effective root drugs, more research is necessary to design more efficient three-dimensional filling systems.

Keywords: *Enterococcus faecalis*, Failed endodontic treatment, Endodontic infection, Culture, PCR

Please cite this article as follows: Hossainpour H, Nazari Z, Moradi P, Vahedian N, Alvandi A, Khazaei S, et al. *Enterococcus faecalis* in untreated root canal teeth: a microbiological study. Avicenna J Dent Res. 2025;17(2):x-x. doi:10.34172/ajdr.2147

Background

Some missteps during procedures, including insufficient disinfectant use for root canal disinfection or instrumentation, can cause a failure in the endodontic treatment. Coronary sealing failure and the complex root anatomy can lead to microorganism microleakage, which could be a significant etiological factor. In these situations, certain Gram-positive bacteria become dominant in root canals. These species can survive in harsh environmental conditions and the minimal presence of nutrients; therefore, they can remain in root canals after root canal treatment. *Enterococcus faecalis* can adhere to root canal walls and endodontic cements, forming a resistant biofilm. Hence, it is usually isolated from root canals in secondary/persistent infection cases (1,2).

Scientific evidence demonstrates that the main causative agents of endodontic therapy failure are microorganisms participating in intra-radicular or extra-radicular infections. Peri -radicular lesions may resist endodontic treatment in the presence of extra-radicular

infection. Intra-radicular infection is undoubtedly a significant risk factor for endodontic treatment failures and can cause root canal treatment failure in two cases. In the first case, microorganisms causing the infection resist the disinfection methods during the root canal treatment process and remain stable. In the second case, microorganisms can infiltrate the root canal system during or after root canal treatment (3,4).

There is a clear distinction between the microbiota of primary intra-radicular infections and secondary or persistent intra-radicular infections associated with endodontic treatment. In contrast to secondary or persistent intra-radicular infections, which usually consist of one or more species of bacteria (typically gram-positive bacteria with no significant predominance between facultative aerobic or anaerobic types), primary intra-radicular infections are normally mixed infections with a predominant population of gram-negative anaerobic rods (5). Obtaining a representative sample from the root canal system can be difficult because of physical constraints. In patients who have undergone retreatment, a lower number of microorganisms are accessible because many of them have been lost during the root canal fill removal process. Thus, obtaining a representative sample from these patients is far more difficult. As a result, the prevalence of a particular species may be underestimated because the number of sampled cells is lower than the detection rate of the identification method (6).

One of the widely used methods for microbial identification purposes is molecular genetic assays, especially polymerase chain reaction (PCR). This is a method with high sensitivity, which makes it a suitable choice for identifying nonculturable microbial species and strains with difficult-to-cultivate characteristics. Although this method has significantly contributed to root microbiota identification in primary infections, it has not been consistently utilized for assessing the prevalence of oral microbes with pathogenic potential in the roots of root-filled teeth with peri-radicular lesions. The biofilm formation capability of *Enterococcus faecalis* has recently been suggested as an important factor in the pathogenesis of *Enterococci* infections (7). Similar to many other pathogenic microorganisms, *E. faecalis* efficiently adheres to biotic and abiotic surfaces, secreting a protective extracellular matrix that leads to the formation of a multilayer antibiotic-resistant biofilm (8,9). Our objective was to detect the presence of *E. faecalis* in teeth with untreated root canals by the molecular assay.

Materials and Methods

Patients and Bacterial Strains

In this cross-sectional study, 32 samples (untreated root canals) were collected from patients aged between 12 years and 70 years at the Dentistry Clinic of the School of Dentistry, Kermanshah University of Medical Sciences (Kermanshah, Iran) from October to December 2023. Clinical procedures were approved by a local ethics committee (IR.KUMS.REC.1400.837), and written informed consent was obtained from each patient. The inclusion criteria were no history of systemic disease, no use of mouth rinses or another preventive measure that can expose the microbial population to antimicrobial agents before sampling, and lack of antibiotic use during the 4 weeks before sampling. The tooth and the surrounding field were then cleansed with 3% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite (NaClO) solution. The coronal restorations were removed after plaque removal, isolation, and disinfection of the operative field. Endodontic access was completed with a sterile high-speed carbide bur until the root filling was exposed. If a post was present, removal was attempted through ultrasonic vibration; if it was unsuccessful, the post was removed with a sterile high-speed carbide bur. After the completion of the endodontic access, the tooth, clamp, and adjacent rubber dam were once again disinfected with 2.5% NaClO. Coronal gutta-percha was removed using sterile Gates-Glidden burs, and the apical material was retrieved using K-type or Edstrom files, or both. Root fillings were removed without the use of chemical solvents. Whenever possible, filling material removed from the canals was transferred to cryotubes containing Tris-ethylenediaminetetraacetic acid (TE) buffer (i.e., 10 mM Tris-HCl and 0.1 mM ethylenediaminetetraacetic acid; pH: 7.6).

Radiographs were taken to ensure the removal of all filling materials. A small amount of the sterile saline solution was then introduced into the root canal by syringe, and the canal walls were filed so that material could be obtained. Initially, the samples were collected using a No. 15 K-type file with the handle cut off. The file was introduced to a level approximately 1 mm short of the tooth apex, determined using diagnostic radiographs, and a discrete filing motion was applied. Afterward, the root canal contents were absorbed into at least 3 paper points. These paper points were transferred to cryotubes containing TE buffer and immediately frozen at 20˚C.

The samples were taken from the untreated root canals of each patient with a sterile tube. After incubation in the brain heart infusion (BHI) medium for 2 hours, the swab was plated on Bile Esculin Agar plates. The plates were incubated aerobically for 48 hours at 37 ˚C. The suspected colonies of enterococci were tested for their positive Gram stain and catalase reaction (Oxoid, Basingstoke, UK).

Biofilm Assay

Biofilm formation for each isolated strain was assessed on polystyrene microplates using the crystal violet staining method with some modifications. Fresh bacterial colonies were inoculated into 5 mL of the BHI broth (Merck, Germany) and incubated at 37 ˚C in an incubator shaker (Precision Thermo Scientific; USA) at a speed of 50 rpm for 4 hours. To confirm growth in the logarithmic phase, the optical density of the suspension was assessed at 625 nm using a spectrophotometer. For biofilm formation,

a bacterial suspension containing 1.5×10^8 CFU/mL was prepared in the BHI broth and subsequently diluted at a ratio of 1:100. A volume of 20 µL/mL of this bacterial suspension was combined with 180 µL/mL of the BHI broth supplemented with 0.25% glucose (BactoTSB medium, BD, Franklin Lakes, NJ, USA) in flat-bottomed 96-well microtiter plates, followed by incubation for 48 hours at 37 ˚C. This procedure was conducted in triplicate for each strain, while pure tryptic soy broth was allocated to 3 wells per plate to serve as negative controls (10,11).

Antimicrobial Assay

Before the antibiotic susceptibility test, *E. faecalis* strains were cultured on a Trypticase soy agar plate and incubated at 35 ˚C for 18–24 hours. The reference strain *E. faecalis* ATCC 29212 was used as a control. Then, a suspension with an inoculum of 10^8 CFU/mL (0.5 standard of the McFarland scale) was prepared from each of the strains, and this suspension was inoculated with sterile swabs on Mueller-Hinton agar plates as a lawn culture. Next, the antibiotic discs were placed on inoculated plates. In this study, the antibiotic susceptibility test for *E. faecalis* isolated was performed using seven antibiotics, including chloramphenicol (30 ug), vancomycin (30 ug), linezolid (30 ug), doxycycline (30 ug), erythromycin (15 ug), ciprofloxacin (5 ug), and ampicillin (10 ug). After 18 hours of incubation at 35 ˚C, the inhibition zone diameters around the antibiotic discs were measured, and the strains were classified as sensitive, moderate, or resistant, according to the Clinical and Laboratory Standards Institute (2023) breakpoints (12).

Deoxyribonucleic Acid Extraction

The root canal samples in the TE buffer were thawed to 37 ˚C for 10 minutes and homogenized by vortex-mixing for 1 minute. The genomic DNA templates were extracted from all culture strains using DNA extraction kits (AccuPrep® Genomic DNA extraction kit; Bioneer, South Korea), according to the manufacturer's instructions. The extracted templates were tested with an ultraviolet spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) at A260/280. Finally, the templates were stored at −20 ˚C for next use.

Polymerase Chain Reaction Assay

PCR was performed using specific primers for the detection of *E. faecalis*; E1, 5'- GTT TAT GCC GCA TGG CAT AAG AG -3' and E2, 5'- CCG TCA GGG GAC GTT CAG -3' (13).

The reaction mixture for the PCR assay was 25 µL and was prepared as 2×Taq premix Master Mix (Ampliqon UK; 12.5 µL), forward and reverse primers (1 µL of each), sterile double-distilled water (7.5 µL), and the DNA sample (3 µL). The DNA amplification for *E. faecalis* samples and positive control (the *E. faecalis* strain ATCC 29212) was performed with the thermal cycle as 95˚C for 2 minutes (the initial denaturation step), 95 ˚C for 30 seconds (the denaturation step), 60˚C (*E. faecalis*) for 1 minute (the primer-annealing step), and 72 ˚C for 1 minute (the extension step). The last three steps were repeated for 36 cycles, and a final step was performed at 72 ˚C for 2 minutes in a thermal cycler (Bio-Rad Thermal Cycler, Bio-Rad Laboratories, Inc., USA).

Data Analysis

The resulting data were entered into a spreadsheet and statistically analyzed using SPSS software (version 22; SPSS Inc., Chicago, IL, USA). Descriptive statistics were applied, and absolute and relative frequencies were calculated.

Results

Identification of Enterococcus faecalis by the Culture Method

Among 32 untreated root canal samples, 6 (18.75%) were identified as *E. faecalis* using the culture method.

Biofilm Assay

Of the 6 isolates, 4 (66.6%) could form biofilms, with 3 (50%) showing weak biofilm ability and 1 (16.6%) representing intermediate biofilm ability.

Antimicrobial Susceptibility Testing

The highest resistance rate among *E. faecalis* isolates was observed against ampicillin (100) and chloramphenicol (83%), respectively. Meanwhile, all isolates were sensitive to vancomycin, and 66.6% were sensitive to linezolid (Table 1).

Polymerase Chain Reaction Assay

Among the 6 *E. faecalis* isolated from root canal samples, 4 (66.67%) were confirmed by PCR (Figure 1).

Discussion

Enterococcus faecalis still has an unknown role in the oral cavity. Although it is not considered the normal flora of the oral cavity, it is associated with some dental diseases, such as peri-implantitis, periodontitis, and dental caries. *E. faecalis* is one of the common bacteria reported in secondary endodontic infections, where it can also cause a biofilm, showing a higher sensitivity of the PCR assay over the culture for detecting *E. faecalis* from root canal samples. It could be related to the detection limits of the technique. For instance, the sensitivity of PCR varies from 10 to 10^2 cells, depending on the technique, and the sensitivity of the culture method is approximately 10^4 to 10^5 cells for target species using nonselective media (14). In addition, PCR is a reliable method for detecting nonviable or viable but nonculturable cells (15).

Based on the findings of a similar study conducted by Hussein et al, 75% of samples were positive for *E. faecalis* by PCR at the molecular level, while with the conventional bacteriological method, this frequency was about 68.8%. In the mentioned study, the result of the antibiotic

Table 1. Antibiotic Resistance Pattern of *Enterococcus faecalis* Isolated From Untreated Root Canal Samples

	C (%)	Van (%)	LZD (%)	D (%)	E (%)	Cip (%)	Amp (%)
Sensitive	17	100	66.6	33.3	16.6	50	0
Intermediate	0	0	16.6	0	16.6	0	0
Resistance	83	0	16.6	66.6	66.6	50	100

Note. C: Chloramphenicol; Van: Vancomycin; LZD: Linezolid; D: Doxycycline; E: Erythromycin; Cip: Ciprofloxacin; AMP: Ampicillin.

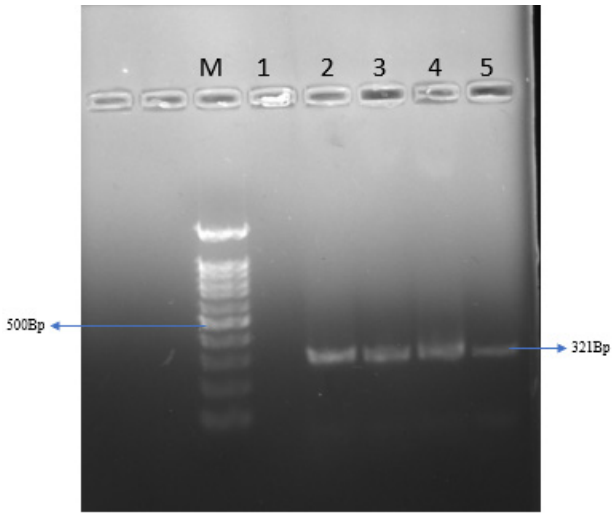

Figure 1. PCR Images of *Enterococcus faecalis* in Root Canal Samples. *Note*. PCR: Polymerase chain reaction. M: Ladder100 Bp; 1: Negative control; 2-4: Root canal samples; 5: Positive control

susceptibility test revealed that all isolates of *E. faecalis* were sensitive to ciprofloxacin, azithromycin, amoxicillin, and vancomycin, and most of them had a moderate sensitivity to co-amoxiclav. Moreover, most isolates were resistant to ceftriaxone, lincomycin, metronidazole, cloxacillin, and cefixime. They also reported that about 90% of the isolates could produce gelatinase, and about 60% of them were able to produce extracellular protease. However, all isolates had biofilm formation ability, and almost all bacteria could adhere to oral epithelial cells (14). In the study by Dumani et al, *E. faecalis* was detected by PCR in 16% and 10% of the necrotic and retreated root canal infections, respectively (13). *E. faecalis* is one of the most common bacteria that can be observed in failed root canal therapy (12%–90%) and early root canal infections in low numbers, especially in teeth with coronal leakage (8,9). Various factors, such as methodologies and geographical influences, can create differences in the composition of root canal microbiota across different studies (10). Several bacteria inhabit the oral cavity, which can contaminate the root canal during endodontic treatment with no sufficient aseptic control or penetrate after root canal treatment via coronal leakage (11). Engström et al found a direct relationship between the existence of enterococci in the oral cavity and that of *Enterococci* in the pulp space (12). Similarly, Dumani et al indicated that *E. faecalis* was detected using culture and PCR assay in 5% and 55% of samples, respectively (13). Viable enterococci usually exist in olives, vegetables, and raw fermented foods, such as meat and cheese (14,15). Recently, some studies based on the next-generation sequencing method have shown an ambiguous composition for endodontic microbiota and have challenged the role of *E. faecalis* as a cause of persistent or secondary root canal infections (16). It is noteworthy that future studies should focus on investigating the relationship between food consumption and the genetic profile of *E. faecalis* strains isolated from the same patient at different times, evaluating the long-term infection of *E. faecalis* in the oral cavity and related risk factors, and examining factors that influence its integration into the oral biofilm (12). The provision of teeth for culture and the isolation of microorganisms for molecular studies were the most important limitations of this study.

Conclusion

Enterococcus faecalis plays a role in the pathogenesis and persistence of apical periodontitis, which was determined by detecting this bacterium in the root canal of a tooth with unsuccessful root canal treatment. This bacterium is one of the common factors in primary and secondary tooth root infections. The survival of *E. faecalis* in the root canals can be due to various factors, including low-efficiency antimicrobial agents, failure in the delivery of irrigating materials and drugs to all parts of the root canal during root canal treatment, incomplete root filling, and failure to achieve a perfect 3D seal, leaving space for bacteria to hide and survive. Therefore, more research is necessary to prepare further efficient antimicrobial agents and more efficient and effective 3D filling systems.

Acknowledgments

The authors gratefully acknowledge the Student Research Center, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.

Authors' Contribution

Design: H. H. and H. M.
Methodology: H. H., N. V., and Z. N.
Writing-original draft: H. M. and H. H.
Writing–review and editing: A. H. A., H. M., and P. M.

Competing Interests

The authors declare no competing interests.

Ethical Approval

The samples (untreated root canals) were collected from the Dentistry Clinic of the School of Dentistry, Kermanshah University of Medical Sciences, Kermanshah, Iran (IR.KUMS.REC.1400.837).

Funding

None.

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