

Analysis of Strain Type and Quantitative of *Enterococcus faecalis* Bacteria in True Combined Endo-Perio Lesions

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Abstract

Enterococcus faecalis bacteria are root canal pathogens that can migrate to periodontal tissue in true combined endo-perio lesions through apical foramen, dentinal tubules, and lateral and accessory canals. The same strain of *E. faecalis* in root canals and pocket periodonsium tissue in true combined endo-perio lesions showed pathological unity and the possibility of migration. The quantity of *E. faecalis* contributes to the disease's severity because of its virulence, i.e., substance aggregation, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase, and hemolysin. To analyze the strain type and quantity of the *E. faecalis* taken from root canals and pockets of true combined endo-perio lesions, primary endodontic lesions, and primary periodontal lesions. 16 samples were taken from the following three diagnosis groups: true combined endo-perio lesions (root canal and pocket), primary endodontic lesions, and primary periodontal lesions. The strain types were analyzed via sequencing methods and quantification real-time PCR. There were similarities of the *E. faecalis* strains in all groups. The largest number was found in the pockets of true combined endo-perio lesions. There were significant differences between the strains in the root canals in true combined endo-perio lesions and those in the primary endodontic lesions. However, there were no significant differences between the strains in the root canals and pockets in true combined endo-perio lesions and between the strains in pockets of true combined endo-perio lesions and in primary periodontal lesions. There were similarities between the strains, and the quantity of *E. faecalis* were also equal between the root canals and the pockets in true combined endo-perio lesions.

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Introduction

A true combined lesion is an endo-perio lesion with inflammation occurring on both the pulp and the periodontium tissue, leading to endodontic infection and periodontium damage or loss of attachment, while also making it difficult to clinically distinguish their major cause.^{1,2} According to Parolia et al., a true combined lesion occurs due to the presence of endodontic disease and a periodontium pocket infection that continues to apex if the degree of attachment loss is large enough.³ Clinically, when performing a pocket probing, the pocket will have a conical

shape toward the apex, and the probe can easily get into the pocket until reaching the pocket's base that is lateral to the root surface, even if extended up to the apex. The radiographic feature of a true combined lesion shows wide radiolucency on the apex that is derived from the endodontic and periodontium.²

E. faecalis is one of the bacteria that cause true combined endo-perio lesions. The size of *E. faecalis* is 0.5-1 μm , while the diameter of dentine tubules is 1-3 μm , allowing the bacteria to penetrate and live on dentine tubules with high pH environments. Furthermore, *E. faecalis* is an anaerobic facultative microorganism; thus, they can live in a deep periodontal pocket. Their size and nature restricts their migration from the periodontium tissue to the root canal, or conversely.⁴ *E. faecalis* forms a biofilm that makes them more resistant to phagocytosis, antibodies, and antimicrobials. They survive from calcium hydroxide through maintaining homeostatic

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pH in passive ways that produce low cytoplasmic membrane permeability. Furthermore, *E. faecalis* has a proton pump that contributes to maintaining homeostatic pH through pumping protons into cells to decrease the internal pH. *E. Faecalis* in the root canal forms colonies on dentin surfaces with the presence of lipoteichoic acid-LTA.^{4,5}

E. Faecalis plays a role in the degree of severity and in the development of periodontitis. *E. faecalis* produces various virulent factors associated with the pathogenesis of periodontitis, including aggregation substance, surface attachment, lipoteichoic acid, and hemolysin that can induce neutrophil dysfunction. Shin et al., state that Gel E had a significant effect on the degradation of the dentine's organic matrix, and the Gel E activity leads to pulp disease and periapical lesions. Virulence is a bacteria's degree of pathogenicity, which can lead to disease within a certain period. A few *E. faecalis* can be eliminated easily; however, if the quantity is greater, they will be difficult to remove and the disease's severity will increase.⁶

Furthermore, *E. faecalis* may collaborate with *F. nucleatum* and increase the depth of the periodontium pocket, the loss of periodontium attachment, and the bleeding upon probing. Therefore, a greater quantity of *F. nucleatum* can support *E. faecalis* colonization on the periodontium environment.⁷ According to Gajan et al., *E. faecalis* was observed in 51.8% of pocket lesions in chronic refractory periodontitis cases.⁸

Numerous researches have already been conducted to identify and quantify the bacteria in root canals and periodontium tissue. However, until now, no studies have discussed the similarities of *E. faecalis* strains and quantities in true combined endo-perio lesion cases compared with primary endodontic lesions and primary periodontal lesions. In this study, *E. faecalis* strains and quantities will be analyzed in root canals and periodontal pocket samples of true endo-perio combined lesions, primary endodontic lesions, and primary periodontium lesions. If the same type of *E. faecalis* exists in these lesions, it could be said that the lesions have pathologic unity, indicating possible *E. faecalis* migration between the root canal and the periodontal pocket, or conversely. The *E. faecalis* strain will be determined using a sequencing method, and

the determination of *E. faecalis* quantity will be done using real-time PCR.

Materials and methods

Sixteen samples from three diagnosis groups (true combined endo-perio lesions, primary endodontic lesions, and primary periodontal lesions) were obtained from root canals and periodontal pockets based on the study's inclusion and exclusion criteria. The inclusion criteria included the age of the subject (19-70 years old), pulp necrosis with a periapical lesion, and pocket depth (≥ 7 mm without recession). If there was recession, the loss of attachment was assessed as the sum of the recession and the pocket's depth (≥ 7 mm). Radiologically, there was bone damage that reached an apex and a diffused periapical lesion with a diameter ≥ 2 mm. Bleeding on probing and the presence of plaque and calculus were local factors. Subjects in this study volunteered and signed informed consent forms. Criteria exclusion included patients with a history of taking antibiotics for any reason within ± 3 months and those patients who refused to participate in the study and who failed to sign an informed consent form.

First, plaque and calculus were cleaned using an ultrasonic scaler. The periodonsium area containing the sample was dried and isolated with a cotton roll. Then, a sterile paper point ISO 40 was inserted into the deepest periodontal pocket for one minute. When taking samples from the root canal, this study used a rubber dam to minimize saliva contamination. After the rubber dam was inserted, the tooth was accessed using a sterile bur and water, a crown-down preparation, and an explorative root canal. A filling motion was performed along the work length until reaching a file with a minimal number 20. For a tooth with multiple roots, samples were obtained from the root with the largest lesion, as seen on the radiographic image. Then, the root canal was irrigated with sterile aquades. A sterile paper point was then inserted on the area nearest the apex to moisten the root canal, and the next sterile paper point, with a minimum ISO 20, was inserted in the root canal for one minute. All samples were placed in a micro centrifuge tube containing 1000ml of PBL and labeled markers. Five μ L samples were taken for a culture medium, and the remaining sample was stored at -20°C until DNA extraction.

The samples' bacteria cultures were performed on chrome agar medium by vortexing samples in a microcentrifuge tube, and then 5 µL of the sample liquid were taken using a pipette and spread on a chrome agar medium and flattened using a slider tool until it covered the entire medium surface. The petri dishes were closed and incubated at 30°C for 24 hours. After 24 hours, bluish green was observed on the chrome agar medium, meaning they were positive for *E. faecalis* colonization. The colony was then cultured with liquid BHI medium and incubated at 37°C for 24 hours. Next, the DNA was extracted using the heat shock method. Spectrophotometry was then performed to determine the DNA's concentration and purity. *E. faecalis* were identified using polymerase chain reaction (PCR) and an electrophoresis technique with agarose from the DNA extract. The primers used in conventional PCR and real-time PCR are:

Primer 16sRNA, Forward: TGG CAT AAG AGT GAA AGG CGC; Reverse: GAC GTT CAG TTA CTA ACG T

Primer Gel E, Forward: ACCCCGTATCATTGGTT; Reverse: ACGCATTGCTTTTCCATC

After the *E. faecalis* were identified, a third person performed the sequencing process, as all samples were sent to Malaysia to obtain the DNA base arrangement for determining the strain type. For determining the *E. faecalis* quantity, real-time PCR was performed on DNA samples that had been stored at -20°C. DNA was then extracted from the samples using the heat shock method and the continued use of the spectrophotometer. Finally, the samples were run using a real-time PCR machine. If all the data were normal for each experiment, a one-way ANOVA analysis was performed using the SPSS 20 software with a significant level of $\alpha = 0.05$. However, if all the data were abnormal, a Kruskal-Wallis analysis was performed

Results

Based on the sequencing method, various DNA base arrangements were obtained (Figure 1). To determine the strain type, a BLAST nucleotide process (Basic Local Alignment Search Tool program) was performed on the DNA base arrangements via BLAST® at the NCBI home page (<http://www.ncbi.nlm.nih.gov/bl->

ast), as shown in figure 2. There was a similarity in the strain samples obtained with ATCC 29 212, showing percentages of 98%, 99%, and 100%. Those percentages were classified into three groups (Table 1): Group A = group that had the same 98% strain with ATCC 29 212 *E. faecalis*; Group B = group that had the same 99% strain with ATCC 29 212 *E. faecalis*; Group C = group that had the same 100% strain with ATCC 29 212 *E. faecalis*.

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>1st_BASE_1_TRUECOMBINED_SAL_AKAR_gelE_Fwd_
CAATGGAATTATGGAACAGATTTAACGGAACATGTCGGTAGTGAAGTAAACGTTAAAAAC
TCTTTTCAAGTAACTTTAATGTCCAGTTGAAAAACAATACGGGAATTGCTTTACACGGGA
ACGGATAACACAGGGGTTTACCATGCAAGTGTGATGGCAAAAATAATTATTCTATTATTCAA
GCACCATCACTAGCGACATTAATCAGAATGCTGTTGACGCCTATACGCATGGAAAATTTGTG
AAAACATATTATGAAGATCATTCCAACGACACAGTATTGATGATCGAGGGATGCCATCTTG
TCAGTTGTTGATGAACAACATCCAGATGCTTATGCAATGCTTTTGGGATGGAAAAGCAAT
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Figure 1. Arrangement of base DNA from the sequencing method.

Figure 1 shows one example of the DNA sequence results, which consists of a nucleotide of DNA sequences (adenine, guanine, cytosine, and thymine) from a DNA molecule taken from a root canal sample of a true combined endo-perio lesion case.

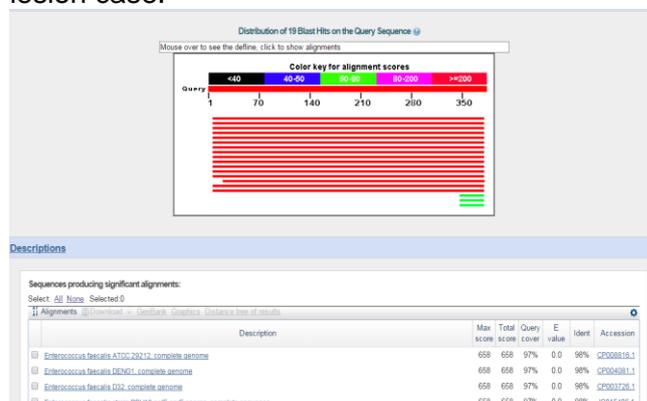


Figure 2. Result of similarity samples' strains compared with ATCC 29212 *E. faecalis* after nucleotide's DNA sequences were blasted.

Figure 2 shows one example of the results determining the similarity strain of samples compared with ATCC 29212 *E. faecalis*. The similarity result was 98%; thus, it was included in group A. Table 1 shows similar strains obtained from the root canals and periodontal pockets of true combined endo-perio lesions belonging to group B. Analysis was performed using real-time PCR to determine the quantity of *E. faecalis* in true combined endo-

perio lesions, primary endodontic lesions, and primary periodontal lesions. A statistical analysis in this study using a Shapiro-Wilk test with a p-value < 0.005 concluded that the data distribution in this study was not normal; thus, a Kruskal-Wallis nonparametric test was performed.

Type of Strains	Root canal of true combined endo-perio lesions	Pocket of true combined endo-perio lesions	Primary endodontic lesions	Primary periodontal lesions
	B	B	B	B

Table 1. Type of strain based on sample source from true combined endo-perio lesions, primary endodontic lesions, and primary periodontal lesions.

Group	N	Median (minimum-maximum)	Mean value±SD	P	
Source of samples	A	4	8.120x10 ¹ (5.436x10 ¹ -0.056x10 ²)	8.059x10 ¹ ±2.531x10 ¹	0.069
	B	4	5.544x10 ⁶ (6.939x10 ¹ -8.9x10 ⁹)	2.253x10 ⁸ ±4.433x10 ⁸	
	C	4	6.922x10 ⁸ (3.672x10 ³ -4.97x10 ⁹)	1.276x10 ⁸ ±2.461x10 ⁸	
	D	4	1.17x10 ² (3.827x10 ¹ -4.037x10 ⁶)	1.009x10 ⁶ ±2.018x10 ⁶	

Table 2. *E. faecalis* quantity based on the sample source of true combined endo-perio lesions, primary endodontic lesions, and primary periodontal lesions. *the limit of significance p < 0.05 using a Kruskal-Wallis test. A = Root canal of true combined endo-perio lesions. B = Pocket of true combined endo-perio lesions. C = primary endodontic lesion. D = primary periodontal lesion

In Table 2, the highest mean value of *E. faecalis* quantity was in the pocket of true combined endo-perio lesions (2.25x10⁸ CFU/ml), followed by primary endodontic lesions, (1.27x10⁸ CFU/ml), primary periodontal lesions (1.01x10⁶ CFU/ml), and finally the root canals of true combined endo-perio lesions (8.06x10¹ CFU/ml). The significance value among the four groups' samples was p > 0.05 (p = 0.069); thus, there was no significant difference in *E. faecalis* quantity among the four groups' samples.

Sample source	Root canal of true combined endo-perio lesions	Primary periodontal lesions
Pocket of true combined endo-perio lesions	0.083	0.149
Primary endodontic lesions	0.021	-

Table 3. Significance value of *E. faecalis* quantity based on the sample source of true combined endo-perio lesions, primary endodontic lesions, and primary periodontal lesions. *the limit of significance was p < 0.05 using a post hoc Mann-Whitney test.

In Table 3, the *E. faecalis* quantity showed a statistically significant difference between the root canals of the true combined endo-perio lesions group and the primary endodontic lesion group, with a p-value < 0.05 (p = 0.021). This means the *E. faecalis* quantity on primary endodontic lesions was greater than on the root canals of the true combined endo-perio lesions. However, there was no significant difference between the pockets of the true combined endo-perio lesions group and the root canals of the true combined endo-perio lesions group, with a p-value = 0.083. There was also no significant difference between the pockets of the true combined endo-perio lesions group and the primary periodontal lesion group, with a p-value = 0.149. This means there was an equal quantity of *E. faecalis* between the pockets of the true combined endo-perio lesions group and the root canals of the true combined endo-perio lesions group, as well as between the pockets of the true combined endo-perio lesions group and the primary periodontal lesions group.

Discussion

This research analyzes the strain types and quantity of *E. faecalis* on true combined endo-perio lesions, primary endodontic lesions, and primary periodontal lesions. *E. faecalis* is an anaerobic facultative gram-positive bacteria with a coccus shape 0.5-1 µm in diameter that can survive in extreme environments, including high alkaline pH and high salt concentrations. These bacteria also colonize in root canals and survive without other bacteria. They grew at 10-45°C and survive at 60°C for 30 minutes. They also have various virulence factors that contribute to the development and severity of pulp diseases and periodontium disease. These factors were

gelatinase (Gel E), aggregate substances (AS), pheromones, and lipoteichoic acid (LTA).^{5,9} In grouping strain types of *E. faecalis* from nucleotides using the blast method, this study observed similar types of strains between the pockets of the true combined endo-perio lesions group and the root canals of the true combined endo-perio lesions group.

Quantification of *E. faecalis* using real-time PCR and statistical analysis shows no significant difference between the number of *E. faecalis* in the root canals and periodontal pockets of true combined endo-perio lesions (Table 3). However, there are differences in the number of *E. faecalis*; the number of *E. faecalis* in pockets is greater than in root canals in true combined endo-perio lesions. This was due to the involvement of the anatomical pathway of the apical foramen, dentine tubules, accessory root canals, and lateral root canals in the true combined endo-perio lesions. *E. faecalis* has a diameter of 0.5-1 μm , while the diameter of dentine tubules is 1-3 μm , thus allowing them to penetrate and live on dentine tubules. Apical foramen, open dentine tubules, and lateral and accessory root canals can lead to possible *E. faecalis* migration from root canals to periodontium tissue, or conversely, in true combined endo-perio lesion cases.

Additionally, the high number of *E. faecalis* in the pockets of true combined endo-perio lesions is due to deep pockets (≥ 7 mm); *E. faecalis* are facultative anaerobic bacteria that can live without oxygen. Deep periodontal pockets have an anaerobic environment and are nutrient-rich, promoting *E. faecalis* colonization. *E. faecalis* can collaborate with other bacteria, such as *F. nucleatum*, in pockets. The high number of *F. nucleatum* can support *E. faecalis* colonization in the periodontium environment. Gajan et al., stated that *E. faecalis* have a 51.8% increased chance of being located on the subgingival in chronic refractory periodontitis cases.⁸ There was a significantly different number of *E. faecalis* between the root canals of true combined endo-perio lesions and those of primary endodontic lesions (Table 3). The number of *E. faecalis* in primary endodontic lesions was greater than in the root canals of true combined endo-perio lesions. This difference is due to the possible migration of *E. faecalis* from root canals to periodontal pockets in true combined endo-perio lesions via anatomic

pathways, which support the smaller *E. faecalis* as compared to dentine tubules. This possible migration leads to a smaller number of *E. faecalis* in the root canals of true combined endo-perio lesions than in primary endodontic lesions.

The number of *E. faecalis* in the periodontium pockets of true combined endo-perio lesions was similar to those between the root canals of true combined endo-perio lesions and primary periodontal lesions. However, the number of *E. faecalis* in the pockets of true combined endo-perio lesions is greater than in primary periodontal lesions. There is a significant number of *E. faecalis* in true combined endo-perio lesions and primary periodontal lesions because the bacteria are anaerobic. In this study, the pocket depth of the true combined endo-perio lesion and the primary periodontal lesion was ≥ 7 mm. Therefore, the second minor hypothesis, which stated there are different numbers of *E. faecalis* between the pockets in true combined endo-perio lesions and primary periodontal lesions, has been rejected.

There was strain similarity between the root canals of true combined endo-perio lesions and those of primary endodontic lesions. Similar results were also found in the pockets of true combined endo-perio lesions and those of primary periodontal lesions (Table 1). These similarities could be due to the *E. faecalis* being derived from the same source. The strain similarity between the pockets of true combined endo-perio lesions could be due to being derived from the same source. The equal quantity of *E. faecalis* between the root canals and pockets of true combined endo-perio lesions should be treated with medication or an irrigation solution that can eliminate the *E. faecalis* from the root canal and the periodontium tissue. According to a previous study, CHX 2% is an effective irrigation solution for eliminating *E. faecalis* from root canals.^{9,10}

Conclusions

There were similar *E. faecalis* strains in true combined endo-perio lesions and in primary periodontal lesions. The number of *E. faecalis* in root canals compared to those in periodontium pockets was equal in true combined endo-perio lesions. The number of *E. faecalis* in root canals of primary endodontic lesions was greater than the number in the root canals of true combined

endo-perio lesions. The number of *E. faecalis* in pockets of true combined endo-perio lesions was equal with those in the pockets of primary periodontal lesions. Further research must be done with a larger number of samples, and the samples should be taken from various places to generalize the strain types in true combined endo-perio lesion, primary endodontic lesion, and primary periodontal lesion cases. Additional research must also be performed on strains from saliva, root canals, and periodontal pockets in true combined endo-perio lesions to determine if the *E. faecalis* was derived from the saliva.

Declaration of Interest

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