PREVALENCE OF *ENTEROCOCCUS FAECALIS* AND *CANDIDA ALBICANS* IN TEETH REQUIRING ENDODONTIC RETREATMENT

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ความชุกของเชื้อเอ็นเทอโรคอคคัส ฟีคาลิส และเชื้อแคนดิดา อัลบิแคนส์ ในฟันที่ต้องได้รับการรักษาคลองรากฟันซ้ำ

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ความล้มเหลวในการรักษาคลองรากฟันมีสาเหตุส่วนใหญ่จากเชื้อโรคที่ยังหลงเหลืออยู่ภายหลังการรักษาคลองรากฟัน มีหลายการศึกษาพบว่าเชื้อ *Enterococcus faecalis* เป็นเชื้อที่พบมากที่สุดในฟันที่ผ่านการรักษาคลองรากฟันและล้มเหลวจากการรักษา แต่ก็มีอีกหลายการศึกษาที่ผลขัดแย้งกัน และมีการศึกษาเมื่อไม่นานนี้พบว่าเชื้อ *Candida albicans* สามารถพบได้ร่วมกับเชื้อ *Enterococcus faecalis* ภายในคลองรากฟันที่มีการติดเชื้อ ในลักษณะ polymicrobial community และเชื้อทั้งสองชนิดยังสามารถสร้างไบโอฟิล์มได้ด้วย ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาความชุกของเชื้อ *Enterococcus faecalis* และ/หรือ *Candida albicans* ในคลองรากฟันที่จำเป็นต้องได้รับการรักษาคลองรากฟันซ้ำ โดยใช้ทั้งเทคนิค culture-based และ polymerase chain reaction (PCR) และศึกษาความสัมพันธ์ระหว่างความชุกของการพบเชื้อกับปัจจัยต่างๆ ทางคลินิก รวมถึงความสามารถในการสร้างไบโอฟิล์มของเชื้อ ซึ่งมีการเก็บตัวอย่างจากคลองรากฟันที่เคยผ่านการรักษาคลองรากฟันมาแล้วและจำเป็นต้องได้รับการรักษาคลองรากฟันซ้ำ ในฟัน 41 ซี่ จากผู้ป่วยจำนวน 35 ราย โดยปัจจัยทางคลินิกซึ่งได้มาจากการตรวจทางคลินิกและภาพถ่ายรังสี ได้แก่ อาการและอาการแสดงทางคลินิก, รอยโรคปลายราก, ขนาดของรอยโรคปลายรากในภาพถ่ายรังสี, คุณภาพของวัสดุอุดคลองรากฟัน และคุณภาพของวัสดุอุดบูรณะบนตัวฟัน การหาความชุกของเชื้อ *Enterococcus faecalis* และ *Candida albicans* ถูกทดสอบทั้งเทคนิค culture-based และ PCR ความสามารถในการสร้างไบโอฟิล์มของเชื้อ *Enterococcus faecalis* จากตัวอย่างทางคลินิก ถูกทดสอบโดยวิธี crystal violet staining assay และคำนวณ percentage เทียบกับ *Enterococcus faecalis* ATCC29212 การวิเคราะห์ความสัมพันธ์ระหว่างความชุกที่พบเชื้อ *Enterococcus faecalis* และปัจจัยต่างๆ ทางคลินิก ประเมินโดยใช้ Fisher's Exact test ผลการศึกษาพบว่าความชุกที่พบเชื้อ *Enterococcus faecalis* คือ 9.8% และ 75.6% เมื่อทดสอบด้วยเทคนิค culture และ PCR ตามลำดับ แต่ไม่พบเชื้อ *Candida albicans* และเมื่อพิจารณาความสัมพันธ์ระหว่างการพบเชื้อ *Enterococcus faecalis* ในคลองรากฟัน ซึ่งทดสอบด้วยเทคนิค PCR กับปัจจัยต่างๆ ทางคลินิก ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) เชื้อ *Enterococcus faecalis* จากตัวอย่างทางคลินิกมีความสามารถในการสร้างไบโอฟิล์มแตกต่างกัน อย่างไรก็ตามเนื่องจากขนาดตัวอย่างที่ทำการศึกษามีจำนวนน้อย จึงไม่สามารถสรุปหรือวิเคราะห์ความสัมพันธ์ระหว่างความสามารถในการสร้างไบโอฟิล์มกับปัจจัยต่างๆ ทางคลินิก ซึ่งควรต้องมีการศึกษาต่อในอนาคต

สาขาวิชา	วิทยาเอ็นโดดอนต์	ลายมือชื่อนิสิต .....
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Sopida Thipung : PREVALENCE OF *ENTEROCOCCUS FAECALIS* AND *CANDIDA ALBICANS* IN TEETH REQUIRING ENDODONTIC RETREATMENT. Advisor: Assoc. Prof. ANCHANA PANICHUTTRA, DDS, PhD. Co-advisor: Assoc. Prof. ORANART MATANGKASOMBUT, DDS, PhD.

Endodontic failures are mainly caused by persistence of microorganisms in the root canal system. *Enterococcus faecalis* has been reported as the most commonly isolated species from root canals with endodontic treatment failure in many studies, but was not the dominant species in others. Recent studies showed that *Candida albicans* is found together with *Enterococcus faecalis* within infected root canals in a polymicrobial community and both species can form biofilm. Therefore, this study aims to investigate the prevalence of *Enterococcus faecalis* and/or *Candida albicans* in root canals requiring retreatment by using both culture-based and polymerase chain reaction (PCR) techniques, and examine its relationship with clinical parameters and the capacity for biofilm formation. Clinical samples obtained from previously filled root canals that required retreatment were taken from 41 teeth (35 adult patients). Clinical parameters, including presence of signs and symptoms, presence of periapical lesion, size of periapical lesion in radiographs, quality of previous root filling and quality of the coronal restoration, were obtained by clinical and radiographical examination. We determined the prevalence of *Enterococcus faecalis* and *Candida albicans* by using both culture-based and PCR. The capacity of biofilm formation of *Enterococcus faecalis* clinical isolates were tested by crystal violet staining assay and calculated into percentage relative to that of *Enterococcus faecalis* ATCC29212. Fisher's Exact test analysis was used to assess relationship between the prevalence of *Enterococcus faecalis* and clinical parameters. The results showed that the prevalence of *Enterococcus faecalis* was 9.8% and 75.6% by culture and PCR, respectively. Our result did not detect any *Candida albicans* in the samples. The Clinical parameters examined were not significantly associated with the presence of *Enterococcus faecalis* (as detected by PCR) in root canals ($p>0.05$). The clinical isolates of *Enterococcus faecalis* showed different levels of biofilm formation. However, due to small sample size, we could not make a conclusion or the association between biofilm formation and clinical parameters which should be studies further.

Field of Study:	Endodontics	Student's Signature .....
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Sopida Thipung

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CHAPTER I INTRODUCTION

The persistence of microorganisms in the root canal system, an enhanced immune inflammatory response, and bone resorption are the main causes of endodontic failure (1, 2). Several studies showed that intraradicular infection of the root canal system is the major causes of the endodontic failure (1, 3, 4). *Enterococcus faecalis* (*E. faecalis*) has been reported as the most commonly isolated species from root canals with endodontic treatment failure in many studies (3, 5-8), but was not the dominant species in others (9-11). In addition, several studies detected *Candida albicans* (*C. albicans*) in root canals of teeth with endodontic treatment failure (3, 7, 9, 12, 13). In a recent study, *C. albicans* is the second most common microbial species (8). *E. faecalis* and *C. albicans* can exist within root canals that are infected in a polymicrobial community and both species can form biofilm. Interestingly, in both nematode infection and in vitro biofilm models, *E. faecalis* has negative impact on virulence of C. albicans. The co-infection caused less pathology and less mortality than infection with either species alone (14-17).

E. faecalis is a Gram-positive, non-spore-forming, fermentative, facultative anaerobic coccus (18). It can form biofilm on root canal dentin, and is resistant to calcium hydroxide; these may be implicated in endodontic treatment failure (2, 19, 20). In vitro studies showed that *E. faecalis* could form biofilm on human dentin in starvation conditions (21). However, no significant relationship was observed between biofilm formation capability of *E. faecalis* strains and sources of isolates from root canals, oral cavity, or other (22). In addition, *E. faecalis* also possess several virulence factors such as, ace, efaA, esp, gelE, asa and asa373 (23). Evidence regarding the prevalence and the factors that are associated with *E. faecalis* in root canal treated teeth are still limited. The factors that have been suggested to influence the presence of *E. faecalis* include signs and symptoms, the presence of apical radiolucent lesion, quality of previous root filling and quality of the coronal restoration (11, 24-26).

In cases of endodontic failure, culture-based and molecular approaches have been utilized to identify microorganisms. Several studies found that microorganisms were

not detected by culture-based techniques in many cases of endodontic failure due to low sensitivity and inability to detect uncultivable phylotypes (18, 27, 28). Meanwhile, polymerase chain reaction (PCR) technique is significantly more effective than culture-based techniques and can identify a higher frequency of *E. faecalis* in the root canal (25, 29-31). This study used both culture-based and PCR techniques to investigate the prevalence of *E. faecalis* and/or *C. albicans* in root canals requiring retreatment, and examined its relationship with clinical parameters and the capacity for biofilm formation.

Research questions

1) What is the prevalence of *E. faecalis* and/or *C. albicans* in root canals requiring retreatment?

2) Is there a relationship between the presence of *E. faecalis* and/or *C. albicans* and clinical parameters, including signs and symptoms, the presence of periapical lesion, size of periapical lesion, quality of previous root filling, and quality of the coronal restoration?

3) Is there a relationship between the presence of *E. faecalis* clinical isolates and the capacity for biofilm formation?

Research objectives

1) To investigate the prevalence of *E. faecalis* and/or *C. albicans* in root canals requiring retreatment.

2) To investigate the relationship between the prevalence of *E. faecalis* and/or *C. albicans* and clinical parameters, including signs and symptoms, the presence of periapical lesion, size of periapical lesion, quality of previous root filling, and quality of the coronal restoration.

3) To investigate the capacity for biofilm formation of *E. faecalis* clinical isolates.

CHAPTER II LITERATURE REVIEW

2.1 Failure of endodontic treatment and microorganisms

The persistence of microorganisms in the root canal system, an enhanced immune inflammatory response, and bone resorption are the main causes of endodontic failure (1, 2). Intraradicular infection is microbial infection of the root canal system during or after treatment due to insufficient aseptic management, poor access cavity design, missing canal, insufficient chemo-mechanical instrumentation, and leaking restorations (temporary or permanent). However, apical periodontitis may persist as asymptomatic radiolucencies after cleaning and shaping because of residual microbial still in the complexity of the root canal system. The complexity of the root canal system is accessory canals, ramifications and anastomoses (1). Two causes of intra-radicular infection are secondary infection and persistent infection. Secondary infection is a new microbial infection of the root canal system during or after treatment procedure, while persistent infection occurs because of microbial resistance to treatment procedure (2). Several studies support that intraradicular infection is the major cause of endodontic failures (1, 3, 4, 32) and the microbial flora in the canal after endodontic failure is different from untreated teeth (4, 32, 33). The microorganisms are mostly Gram-positive facultative anaerobic bacteria, especially *E. faecalis* (3, 4, 7).

E. faecalis is a Gram-positive, non-spore-forming, fermentative, facultative anaerobic coccus (18). It is a commonly isolated species from failed root canal treatment because of its biofilm-forming abilities in root canals that is resistant to intracanal medication (2, 19) and its ability to penetrate into dentinal tubules to escape the action of irrigants during chemomechanical endodontic treatment (34, 35). *E. faecalis* is also resistant to calcium hydroxide at high pH because of the activity of proton pump that drives protons to maintain the cytoplasmic pH. and may be found as a single infection in root canals (4). It has survival mechanism when exposed to unfavorable environmental conditions such as low nutrient concentrations, low or high temperatures, excessive salinity, and pH extremes. It can be in a viable but noncultivable (VBNC) state on growth

media, pathogenicity viability and pathogenicity must both be maintained, which can be restored in favourable environmental conditions (36, 37). However, in persistent extra-radicular infections, *Actinomyces israelii* and *Propionibacterium propionicum* is also prevalent (38). By culturing technique, Sundqvist and colleagues investigated the composition of the microbial flora in 54 root-filled teeth with persisting periapical lesions. They found that the bacterial species recovered from 24 of 54 canals after removal of the previous root filling and *E. faecalis* was the most commonly isolated species (38%) (4). Molander and colleagues examined the microbiological status of 100 root-filled teeth with apical periodontitis. They found that the bacterial species recovered from 68 of the 100 teeth and *E. faecalis* was the most commonly isolated species (47%) (3). Peciuliene and colleagues investigated the occurrence of *E. faecalis* in 25 root-filled teeth with asymptomatic with apical periodontitis in Lithuanian patients. They found that the bacterial species recovered from 20 of the 25 teeth and *E. faecalis* was the most commonly isolated species (70%) (5). Then, Hancock and colleagues investigated the composition of the microbial flora in 54 root-filled teeth with persistent periapical radiolucencies in North American population. They found that the bacterial species recovered from 34 of the 54 teeth and *E. faecalis* was the most commonly isolated species (30%) (6). Pinheiro and colleagues investigated the microbial flora in 60 root-filled teeth with periapical lesions. They found that the bacterial species recovered from 51 of the 60 teeth and *E. faecalis* was the most commonly isolated species (53%) (7). In addition, Pourhajibagher and colleagues investigated the microorganisms associated with primary and secondary endodontic infections by culture, biochemical tests, and molecular method in an Iranian population. They found that *Veillonella parvula* (20.6%) and *E. faecalis* (36.6%) was most frequently found in primary and secondary endodontic infections, respectively (8). But some studies have demonstrated that *E. faecalis* is not as the main microorganism in root canal treated teeth (9-11). Rocas and colleagues evaluated the presence and relative levels of 28 bacterial taxa in 17 root-filled teeth with apical periodontitis in German patients by reverse-capture checkerboard hybridization. They found that mixed infection (9). Rocas and colleagues also investigated by using the reverse capture-checkerboard DNA

hybridization and quantitative real-time PCR (qPCR) technique. They found that the most prevalent taxa detected by checkerboard was *Propionibacterium* species, *Fusobacterium nucleatum*, Streptococci, and *Pseudoramibacter alactolyticus*. *E. faecalis* and Streptococci was detected by qPCR 38% and 41%, respectively (10). Murad and colleagues investigated the microbiota in 36 root-filled teeth with periapical lesion. They found that mixed infection with *Enterococcus faecium* and *Streptococcus epidermidis* was the most highly prevalent species (36%) (11). However in recent years, Next-generation sequencing (NGS) has been used to assess bacteria diversity in root canals (39, 40). A systematic review of the root canal microbials reported that main species detected in root canal treated teeth are Fusobacterium, Actinomyces, Porphyromonas, Prevotella and Streptococcus (41, 42). In addition, several studies reported that *C. albicans* is found in root canals of teeth with endodontic treatment failure (3, 7-9, 12, 13, 43).

C. albicans is a dimorphic fungus, including yeast and filamentous forms (hyphae). The fungus uses hyphae to colonize and penetrate host tissues (44). *C. albicans* can be found in both monomicrobial and polymicrobial infections suggesting it may play a function in the pathogenesis of endodontic infections (17). Moreover, It is commonly detected in canals after endodontic treatment failure because it can resist calcium hydroxide and penetrate into dentinal tubules, this may explain why *C. albicans* has been linked to cases of persistent root canal infections (45, 46). *C. albicans* can also form biofilm to resist to antifungal agents (47). Several studies reported that *C. albicans* is detected in the root canals of teeth that have failed to respond to endodontic treatment (3, 4, 7, 9, 12, 13). By culturing technique, Sundqvist and colleagues investigated the composition of the microbial flora in 54 root-filled teeth with persisting periapical lesions and found 8% *C. albicans* (4). Molander and colleagues investigated the microbiological status of 100 root-filled teeth with apical periodontitis with 4% *C. albicans* (3). Pinheiro and colleagues investigated the microbial flora in 60 root-filled teeth with periapical lesions. They found that the prevalence of 3% *C. albicans* (7). Rocas and colleagues evaluated the presence and relative levels of 28 bacterial taxa in 17 root-filled teeth with

apical periodontitis in German patients. They found 6% *C. albicans* by PCR technique (9). Poptani and colleagues investigated prevalence of *C. albicans* in 20 root filled teeth symptomatic with chronic apical periodontitis with or without periradicular lesions and 35% *C. albicans* (12). Then, Persoon and colleagues systematically reviewed the literature on the prevalence and diversity of the fungi found in root canal infections. They found that fungi have been isolated from up to 7.5% of root canal infection and *C. albicans* is the most prevalent species (13). In a recent study, Pourhajibagher and colleagues investigated the microorganisms associated with primary and secondary endodontic infections by culture, biochemical tests, and molecular method in an Iranian population. They found that *C. albicans* was the second most common species (20%) in secondary endodontic infections (8)

Recently, a number of studies investigated the interaction and co-existance of *E. faecalis* and *C. albicans*. In Caenorhabditis elegans model of co-infection, *E. faecalis* was able to prevent *C. albicans* from forming hyphal morphogenesis using the Fsr quorum-sensing system. *E. faecalis* uses small peptides, the gelatinase biosynthesis-activating cluster (GBAP) peptide, to activate the Fsr quorum sensing system via the FsrB transcriptional regulator. In *E. faecalis*, FsrB is a key virulence regulator. The inhibition of hyphal formation may be caused by GBAP's direct action on fungal cells or via Fsr regulated genes (15, 16). The transformation from yeast to hyphal form is required for *C. albicans* to escape phagocytic cells, so the inhibition hyphal morphogenesis by *E. faecalis* may inhibit virulence of *C. albicans* (15-17, 48). Graham and colleagues showed that treatment with 0.1 nM of EntV, a bacteriocin produced from *E. faecalis*, completely inhibit killing by *C. albicans* in nematodes (14). Macrophages are protected by EntV, which increases their antifungal action. In a murine model of oropharyngeal candidiasis, EntV also reduces epithelial invasion, inflammation, and fungal load. In all models, it reduce the number of fungal cells present in the hyphal form. So, EntV is a specific inhibitor that can reduce *C. albicans* virulence and biofilm formation at subnanomolar concentrations through the inhibition of hyphal formation (14). In contrast, *E. faecalis* is more resistant to starvation and has improved survival rates in the presence of *C. albicans*

when biofilm is observed on the root canal dentin by using scanning electron microscopy. This may be because the decomposition of *C. albicans* may provide some essential nutrients for *E. faecalis* survival under starvation (49). Msb2, a membrane protein produced by *C. albicans*, has the ability to bind and inactivate host defense proteins so subsequently provide the protection to *E. faecalis* from host defence mechanism and leading to long-term colonization of *E. faecalis* in the root canal (17). Thus, the dynamic interaction between *E. faecalis* and *C. albicans* may be important for endodontic treatment outcomes, but further investigations are needed.

2.2 Identification microorganisms by culturing and molecular techniques

Endodontic bacteria have traditionally been investigated using culture-based techniques, which rely on isolation, growth, and identification in the lab using morphological and biochemical testing. However, these techniques have several limitations, including time-consuming, low diagnostic sensitivity, and the inability to cultivate a large number of bacterial as well as misidentification of atypical bacteria. Molecular techniques, such as polymerase chain reaction (PCR), allow the identification of uncultivable bacteria and cultivable atypical bacteria that cannot be accurately identified by culture-based techniques. The molecular technique has the advantages of detecting both cultivable and uncultivable microbial species, higher specificity, direct detection of microbial species in clinical samples without cultivation, higher sensitivity, less time-consuming, does not require carefully controlled anaerobic conditions during sampling and transportation, and when there are a lot of samples, they can all be kept and evaluated at the same time. The PCR process uses denaturation, primer annealing, and extension cycles to replicate DNA. The template DNA melts at high temperatures, breaking the hydrogen bonds and forming single strands of DNA. Two short oligonucleotides (primers) are annealed on the target DNA's opposing strands. As a result, new DNA products are exponentially multiplied. Then, the PCR amplicons will be separated by gel electrophoresis and move through the gel according by size. Ethidium bromide staining and UV transillumination are commonly used to view the gel. Positive

PCR result is PCR amplicon of the predicted size match with designed primers (18, 27, 28).

2.3 The relationship between the presence of *E. faecalis* and clinical parameters

The relationships between the prevalence of *E. faecalis* and clinical parameters in root-filled teeth has only a small number of studies examined and the results are still inconclusive (11, 24-26). Kaufman and colleagues showed that the presence of periapical lesion significantly correlated with the presence of *Enterococcus* spp. (24). Zoletti and colleagues found that there was no significant difference between the occurrence of *E. faecalis* with and without periapical lesions (25). In addition, Wang and colleagues and Murad and colleagues found that the presence of *E. faecalis* in root canals was not significantly associated with the tooth location, presence of clinical symptoms, type of coronal restoration, restoration status, number of root canals, or canal obturation quality. However, higher counts of gram-negative rod species were associated with periapical lesions with the largest areas (11, 26). Nevertheless, clinical parameters that may influence the prevalence of *E. faecalis* can influence endodontic success or failure of endodontic retreatment. Previous studies suggested that quality of the previous root filling, the occurrence of a perforation, and apical periodontitis are significant outcome predictor of treatment success (50, 51).

2.4 The capacity for biofilm formation of *E. faecalis*

In the root canal system, *E. faecalis* can produce various virulence factors that enhance adhesion, colonization and biofilm formation. *E. faecalis* virulence genes found in teeth after endodontic treatment failure include *ace, efaA, esp, gelE, asa* and *asa373* (23) but the *ace* genes do not appear to involve in biofilm formation in primary infection (52). The *ace* gene is collagen binding protein important in adhesion and the most prevalent in root canal treated teeth. The *efaA* gene is endocarditis antigen. The asa and asa373 genes are aggregation substance, while *esp* encodes a surface protein that may be involved in *E. faecalis* colonization and persistence. The *gelE* gene encodes a gelatinase that may aid in host immune evasion (23). Several studies investigated the

capacity of biofilm formation of *E. faecalis* clinical isolates. Anderson and colleagues investigated correlation between presence of virulence genes (*asa1, cylA,* and *esp*) and biofilm formation capacity of *E. faecalis* clinical isolates from four origins (food, clinical specimens, root canal treated teeth and plaque/saliva). They found that *cylA* and *esp* gene correlated significantly with the origin of *E. faecalis* clinical isolates. The cylA and esp-positive isolated from plaque/saliva samples. Whereas root canal treated teeth revealed a lower abundance of *cylA* and *esp* than all the other isolates. The 56.5% of cylA-positive isolated from plaque/saliva samples and 51.0% of the cylA-negative isolated from endodontic samples. The 51.6% of esp-positive isolated from plaque/saliva samples and 50% of esp-negative isolated from endodontic samples ($p<0.001$). In addition, The presence of *asa1* was associated with moderate biofilm formation capacity (53). Zheng and colleagues found that *cylA* was associated with weak biofilm formation ($0.5<$ OD570 <1), while and *esp* was associated with strong (OD570 >2) or medium (OD570, 1–2) biofilm formation (54). Yoo and colleagues investigated correlation between biofilm formation and extracellular material (ECM) levels of *E. faecalis* clinical isolates. ECM surrounding *E. faecalis* might increase resistance to environmental stresses. They found that all isolates produced ECM in an aerobic environment and strong biofilm producers (OD570 nm$\geq$ 0.55) could upregulate ECM production. However, there was no association between the presence of *esp* or gelatinase activity and ECM synthesis (55).

Biofilm morphologic structure vary and has no unique pattern for endodontic infections (56). Histologically, clinical strains of bacteria in root canal treated teeth with apical periodontitis are in form of biofilms both in symptomatic and asymptomatic group and lead to endodontic treatment failure (57). In symptomatic teeth, bacteria usually form biofilm in large colonies but in asymptomatic teeth usually form in small colonies (57). In addition, biofilms are significantly associated with large epithelialized lesions (cysts and epithelialized granulomas or abscesses) but no significantly associated with clinical symptoms (56).

CHAPTER III MATERIALS AND METHODS

3.1 Study population

The study included thirty-five adult patients (forty-one teeth) who attended the Department of endodontics, Faculty of dentistry, Chulalongkorn University, for non-surgical endodontic retreatment. The study protocol was approved by the ethics committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand (ref.032/2019) in accordance with the Helsinki Declaration. Before the sample collection, all participants signed a written informed consent form.

Inclusion criteria included patients with previously root-filled single root canal or multirooted teeth with failed treatment at first or second times based on clinical and radiographic investigations with at least one of the following: persistent signs and/or symptoms, such as pain on palpation, pain on percussion, swelling, pus, or sinus tract opening; radiographic evidence of larger periapical lesion or persistent periapical lesion (endodontic therapy completed more than 4 years); and unsatisfactory root canal obturation, such as underfilled > 2 mm. from the radiographic apex, overfilled, had void, or missed canal in cases required new prosthetic restoration. Exclusion criteria included patients who had antibiotic treatment in the previous three months, tooth with periodontal diseases or had probing depth of > 5 mm, and extreme loss of tooth structure such that the tooth could not be isolated with a rubber dam.

Sample size calculation

The sample size for this study was calculated based on the prevalence of *E. faecalis* in failed endodontic cases by PCR technique according to a previous study (58), which was 77%. When using the following formula with error (d) = 0.10, Alpha (α) = 0.05, Z(0.975) = 1.95996, the sample size was 69.

$$n = \frac{z^2_{1-\frac{\alpha}{2}}\, p(1-p)}{d^2}$$

However, due to the limitations on the availability of the patients who qualified the eligibility criteria, 41 teeth were enrolled for this study. This resulted in an increase in error rate from 10 to 13%.

3.2 Data collection

Medical and dental history were collected from hospital records, interviews, and clinical and radiographical examinations. The following variables were recorded for each patient: 1) age 2) gender 3) type of tooth 4) absence or presence of the clinical signs and symptoms defined as moderate to severe pain on percussion or palpation or any flare-up 5) absence or presence of periapical lesion 6) size of periapical lesion (the largest dimension in mm.) 7) quality of previous root filling which were classified into acceptable and unacceptable (when root canal filling was underfilled > 2 mm. from the radiographic apex, overfilled, had void, or missed canal) 8) quality of the coronal restoration which were classified into intact and defective. The intact coronal restorations had adequate seal both clinically and radiographically.

Because of, the definition of success and failure of endodontic treatment differed from the goal of treatment. These were elimination apical periodontitis, prevention of apical periodontitis, or absence of clinical presentation and functional. Therefore, the outcomes of endodontic treatment should be defined in healing and disease. In this study, we classified the healing of endodontic treatment which modified from Friedman and colleagues into healed and not healed (59). In healed group defined as absence both of the clinical signs and symptoms and periapical lesion. In not healed group defined as presence of clinical signs and symptoms and/or presence of periapical lesion.

3.3 Sampling procedures

Sample collections was as described with minor modifications (58). During endodontic procedures, aseptic techniques were used. A rubber dam was used to separate each tooth, which was then disinfected with 1.5% tincture iodine, followed by 70% alcohol. Access preparation was carried out using sterile burs with only normal saline solution for irrigation until the root filling was exposed. Coronal gutta-percha was removed

by using sterile gate-glidden burs and the apical material was removed by using K-files or H-files or both (Dentsply Maillefer, Ballaigues, Switzerland) without using any chemical solvent. Apex locator and radiography were used to determine working length (Dentaport Root ZX, J Morita , Irvine, CA, USA). Then, the root canal wall was filed by using K-files (Dentsply Maillefer, Ballaigues, Switzerland) at working length to at least size 25. At least 3 sterile paper points were introduced to the working length (a level approximately 0.5 mm. short of the root apex) for 60 seconds each. One of these paper points was collected in cryotubes containing TE buffer (ie, 10 mM Tris-HCl and 0.1 mM EDTA; pH, 7.6), placed on ice, and transferred to the laboratory for PCR analysis. While another one was transferred into brain heart infusion broth (BHI broth; HiMedia Laboratories, Mumbai, India) for culture.

3.4 Culture techniques for *E. faecalis* identification

The samples in BHI broth were vortexed and the bacterial suspension was plated on Mitis Salivarius Agar (MSA; DifcoTM Mitis Salivarius Agar, Becton, Dickinson and Difco, Chicago, IL, USA) and incubated at 37°C for 24 hours. From each plate, dark blue colonies with smooth surfaces were presumed to be *E. faecalis* and were subcultured. Gram's staining and biochemical tests were used to characterize the isolated pure cultures. The biochemical test using sorbitol fermentation and *Streptococcus faecalis* (SF) broth (reagents from HiMedia Laboratories, Mumbai, India).

3.5 Culture techniques for *C. albicans* identification

The samples in BHI broth were vortexed and was plated on Sabouraud dextrose agar (HiMedia Laboratories, Mumbai, India) with antibiotics (Penicillin G sodium and Streptomycin) and incubated at 30°C for 48 hours. The colonies were stained with Gram staining to examine the organisms. Chromogenic candida agar (CHROMagar, Paris, France) was used for preliminary species identification. Colony colour and morphology were evaluated after 48 hours. Polymerase chain reaction (PCR) was used for confirmation (60)

3.6 Polymerase chain reaction techniques

The root canal samples in TE buffer were thawed to 37°C for 10 minutes and vortexed for 1 minutes. The pellets were collected by centrifugation, washed 3 times with 200 µL of MilliQ water, and resuspended in 200 µL of MiliQ water. Then, samples were boiled for 10 minutes, quickly chilled on ice for 5 minutes, and centrifuged at 4°C to remove unbroken cells and large debris. The supernatant was collected and used as a PCR amplification template (58).

PCR was performed as previously described using *E. faecalis* species-specific primers (EFLF (5'-GTT TAT GCC GCA TGG CAT AAG AG-3' GenBank accession no. Y18293) and EFLR (5'-CCG TCA GGG GAC GTT CAG-3' GenBank accession no. Y18293) which produce a PCR amplicon of 310 bp (18, 58); *C. albicans* specific primers (CAL5 (5'-TGTTGCTCTCTCGGGGGCGGCCG-3') and NL4CAL (5'-AAGATCATTATGCCAACATCCTAGGTAAA-3')) which amplify a 175-bp DNA fragment (60); and panfungal primers (ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3′)) which amplify a 500-800 bp DNA fragments) (61-63). PCR products of *C. albicans* using ITS1 and ITS4 primers were approximately 530 bp (64). PCR conditions for each primer set were as previously described.

3.7 Biofilm formation

Biofilm formation assay was performed as described (22, 65, 66). *E. faecalis* (ATCC29212) and clinical isolates were cultivated overnight in Tryptic Soy Broth (TSB) at 37 °C and were adjusted to OD_{600} of 0.1. The cultures were incubated until to log phase (OD_{600} = 0.4-0.6) and were adjusted to OD_{600} of 1.4 (approximately 10^7 CFU/ml). Then, The cultures were diluted 1 : 100 in 200 µl of TSBG (TSB with 0.25% glucose) and inoculated into 96-well polystyrene microtiter plates at 37 °C for 24 hours. Two wells per strain were inoculated. TSB with 0.25% glucose was negative controls. After 24 hours, the supernatant was carefully discarded by using a micropipette, and plates were washed with distilled water to remove unattached cells. Biofilms were fixed with 100 µl of 70% methanol for 30 minutes, stained with 100 µL of 1% crystal violet solution in water for 15

minutes, and wells were washed with distilled water. Each well was added with 100 µL of 30% acetic acid to destain the biofilms for 10 minutes. The optical density at 570 nm (OD570) was measured by using a microtiter plate reader (Bio-Tek, USA). Each assay was performed three times.

3.8 Statistical Analysis

The capacity of biofilm formation was calculated into percentage of biofilm mass of each clinical isolate relative to that of *E. faecalis* ATCC29212. Chi-square or Fisher's Exact Test analysis was used to assess relationship between the prevalence of *E. faecalis* and clinical parameters, including signs and symptoms, presence or absence of periapical lesion, size of periapical lesion, quality of previous root filling, and quality of the coronal restoration. All analyses were performed with IBM SPSS statistics for windows, version 22.0 (IBM, Armonk, NY). A P-value < 0.05 was considered statistically significant.

CHAPTER IV RESULTS

A total of 41 samples were collected from 35 patients, 9 males and 26 females, with an age range of 19-74 years (mean, 49.4 ± 17.3 years). Among the 41 samples, 25 were anterior teeth, 13 were premolars and 3 were molars. The teeth were diagnosed as previously treated teeth with normal apical tissue (22 teeth), asymptomatic apical periodontitis (15 teeth), symptomatic apical periodontitis (3 teeth) or chronic apical abscess (1 teeth). The reasons for retreatment were persistent signs and/or symptoms (4 teeth), such as pain on palpation, pain on percussion, or sinus tract opening; radiographic evidence of persistent periapical lesion or larger periapical lesion (17 teeth); unsatisfactory root canal obturation (33 teeth), such as underfilled > 2 mm. from the radiographic apex, had void, or missed canal; and unsatisfactory coronal restoration (21 teeth), such as leakage in cases required new prosthetic restoration.

4.1 The prevalence of *E. faecalis* and/or *C. albicans*

The prevalence of *E. faecalis* was 9.8% (4 in 41 teeth) and 75.6% (31 in 41 teeth) by culture and PCR techniques, respectively. We did not detect any *C. albicans* in the samples we examined.

4.2 The relationship between the prevalence of E. faecalis and clinical parameters

The relationship between *E. faecalis* (as detected by PCR) and clinical parameters was analyzed. (Table 1) Clinical parameters, including presence of signs and symptoms, presence of periapical lesion, size of periapical lesion in radiographs (in cases with periapical lesion, N=17), quality of previous root filling, quality of the coronal restoration, and healing of endodontic treatment were not significantly associated with the presence of *E. faecalis* in root canals ($p > 0.05$). *E. faecalis* was detected in only four samples using culture methods. None of the patients had any clinical signs and symptoms, but all of the teeth had inadequate root canal filling (underfilled > 2mm. and had void). Two of these samples had periapical lesions of 3 mm. and 7 mm. in diameter. Three of these samples had defective coronal restorations (leakage). Two of these samples had healing of endodontic treatment (absence of clinical signs and symptoms and periapical lesion).

Clinical characteristics were presented in table 2 using PCR methods. The comparison between the presence of *E. faecalis* and healing, The presence of *E. faecalis* in root canals could healed, though the samples had unacceptable root canal obturation or defective coronal restoration. The absence of *E. faecalis* in root canals in healed group, samples had unacceptable root canal obturation and could be both intact and defective coronal restoration. However, the presence of *E. faecalis* in root canals (80.6%) was isolated from a tooth with unacceptable of quality of previous root filling. Interestingly, the presence of *E. faecalis* in root canals in not healed group, samples mostly had no signs and symptoms, periapical lesion size < 5 mm. and unacceptable root canal obturation. But although *E. faecalis* was not found, some of samples could not healed. These be found in both in acceptable or unacceptable root canal obturation.

Table 1 Relationship of prevalence of *E. faecalis* and clinical parameters

	E. faecalis in root canals (by PCR)		P value[a]	*E. faecalis* in root canals (by culture)
	Yes(n=31) %(n)	No(n=10) %(n)		Yes (n=4) %(n)
Clinical signs and/or symptoms				
Yes (n=4)	6.5 (2)	20 (2)	0.245	0 (0)
No (n=37)	93.5 (29)	80 (8)		100 (4)
Periapical lesion				
yes (n=17)	48.4 (15)	20 (2)	0.152	50 (2)
No (n=24)	51.6 (16)	80 (8)		50 (2)
Size of periapical lesion(N=17)				
< 5 mm (n=13)	73.3 (11)	100 (2)	1.000	50 (1)
≥ 5 mm (n=4)	26.7 (4)	0 (0)		50 (1)
Quality of previous root filling				
acceptable (n=8)	19.4 (6)	20 (2)	1.000	0 (0)
unacceptable (n=33)	80.6 (25)	80 (8)		100 (4)
Quality of the coronal restoration				
intact (n=20)	41.9 (13)	70 (7)	0.159	25 (1)
defective (n=21)	58.1 (18)	30 (3)		75 (3)
Healing of endodontic treatment				
healed (n=22)	51.6 (16)	60 (6)	0.727	50 (2)
not healed (n=19)	48.4 (15)	40 (4)		50 (2)

[a] Fisher's Exact Test

Table 2 Relationship of clinical characteristics and prevalence of *E. faecalis* using PCR methods

E. faecalis (n)	Healing (n)	Signs and/or symptoms (n)	Periapical lesion (n)	Size of lesion (n)	Quality of previous root filling (n)	Quality of the coronal restoration (n)	Type of tooth* (n)
Detected (31)	Healed (16)	Yes (0)	-	-	-	-	-
		No (16)	Yes (0)	-	-	-	-
			No (16)	-	Acceptable (3)	Intact (1)	P (1)
						Defective (2)	A (1), P (1)
					Unacceptable (13)	Intact (5)	A (3), P (1), M (1)
						Defective (8)	A (4), P (3), M (1)
	Not Healed (15)	Yes (2)	Yes (2)	<5 mm. (0)	-	-	-
				≥5 mm. (2)	Acceptable (1)	Intact (1)	A (1)
						Defective (0)	-
					Unacceptable (1)	Intact (1)	A (1)
						Defective (0)	-
			No (0)	-	-	-	-
		No (13)	Yes (13)	<5 mm. (11)	Acceptable (2)	Intact (0)	-
						Defective (2)	A (2)
					Unacceptable (9)	Intact (5)	A (2), P (3)
						Defective (4)	A (2), P (2)
				≥5 mm. (2)	Acceptable (0)	-	-
					Unacceptable (2)	Intact (0)	-
						Defective (2)	A (2)
			No (0)	-	-	-	-

Table 2. (cont.) Relationship of clinical characteristics and prevalence of *E. faecalis* using PCR methods

E. faecalis (n)	Healing (n)	Signs and/or symptoms (n)	Periapical lesion (n)	Size of lesion (n)	Quality of previous root filling (n)	Quality of the coronal restoration (n)	Type of tooth* (n)
No Detected (10)	Healed (6)	Yes (0)	-	-	-	-	-
		No (6)	Yes (0)	-	-	-	-
			No (6)	-	Acceptable (0)	-	-
					Unacceptable (6)	Intact (3)	A (2), P (1)
						Defective (3)	A (3)
	Not Healed (4)	Yes (2)	Yes (0)	-	-	-	-
			No (2)	-	Acceptable (1)	Intact (1)	P (1)
						Defective (0)	-
					Unacceptable (1)	Intact (1)	M (1)
						Defective (0)	-
		No (2)	Yes (2)	<5 mm. (2)	Acceptable (1)	Intact (1)	A (1)
						Defective (0)	-
					Unacceptable (1)	Intact (1)	A (1)
						Defective (0)	-
				≥5 mm. (0)	-	-	-
			No (0)	-	-	-	-

* A=Anterior, P=Premolar, M=Molar

4.3 The relationship between the prevalence of E. faecalis isolates and the capacity for biofilm formation.

To investigate their capacity for colonization, the four *E. faecalis* isolates obtained from the root canals were tested for biofilm formation by crystal violet staining assay. The percentage of biofilm mass formed by each of these isolates relative to that of *E. faecalis* ATCC29212, a standard laboratory strain control, is shown in Figure 1. Among these isolates, E3 had the highest biofilm mass (35.88%±16.5%), followed by E1 (27.40%±7.1%), while E2 and E4 showed similar levels of biofilm formation (10.27%±2.8%, 10.13%±4.7%, respectively). Interestingly, the isolate with the highest biofilm formation, E3, was the only one isolated from a tooth with intact coronal restoration but unacceptable of quality of previous root filling (void) (Table 1).

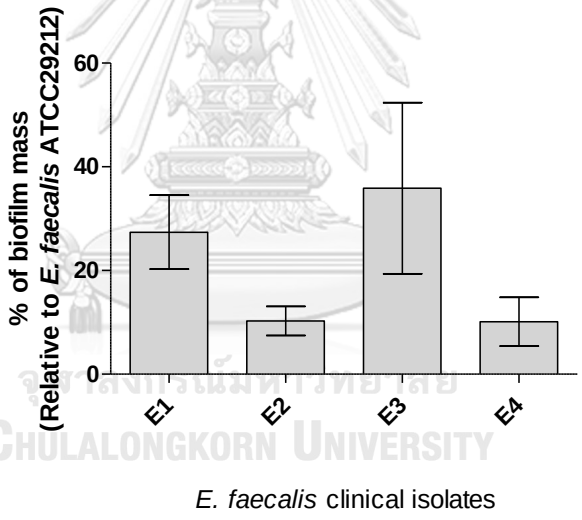

Figure 1 Average percentage of biofilm formation of *E. faecalis* clinical isolates (E1-E4) relative to that of a standard laboratory strain (ATCC29212).

CHAPTER V DISCUSSION AND CONCLUSION

In this study, we examined 41 teeth requiring endodontic retreatment for the prevalence of *E. faecalis* and/or *C. albicans* in the root canals. We did not detect *C. albicans* in any of the samples examined. We found that PCR technique could detect *E. faecalis* approximately 8 times as efficient as culture-based method. We detected *E. faecalis* in 9.8% and 75.6% of endodontically treated teeth requiring retreatment, by culture and PCR techniques, respectively. Due to limited availability of patients, we were able to enrolled only 41 teeth in this study; this increased the error rate of the estimation (d) from 0.10 to 0.13. Previous studies reported the prevalence of *E. faecalis* in root canals with endodontic treatment failure and persistent intra-radicular infection ranging from 30% to 76% (4-8, 29). By culture technique, the previous studies included only root canal treated teeth with periapical lesion (4-7) and asymptomatic teeth (4, 5). But in our study, we examined both root canal treated teeth with or without periapical lesion and asymptomatic or symptomatic teeth. Gomes and colleagues showed that the prevalence of *E. faecalis* was detected as frequently in root canal treated teeth with periapical lesion when a nPCR (nested amplification with species-specific 16S primers) analysis was used compared with culture technique (29). In addition, Pourhajibagher and colleagues found that the prevalence of *E. faecalis* was 36.6% in secondary endodontic infections by 16S ribosomal RNAgene sequencing (8). Correspond to Dioguardi and colleagues found that *E. faecalis* was detected as frequently in persistent intra-radicular infections (38). However, the new techniques which used detected of microorganisms determine more microbial composition and diversity involved in endodontic disease (41-43). The presence of *E. faecalis* in root canals 80.6% was isolated from a tooth with the unacceptable root canal obturation while 19.4% was isolated from a tooth with the acceptable root canal obturation. These results may demonstrate that the importance of root canal treatment procedures in reducing numbers of residual microorganisms in root canal. Although, several studies showed that instrumentation and medication cannot completely clear microbial infection of the root canal system after root canal treatment (67-79). The residual

microorganisms have decrease in numbers and types but they may be the cause of secondary and persistent endodontic infection (1). These microorganisms are able to attach to the root canal wall and form biofilm in ramifications, lateral canals, isthmuses, irregularities areas or dentinal tubules that are impossible to reach with the instrumentation (19, 57, 80, 81). These bacteria are also able to adapt gene expression to survive in low nutrient conditions (4, 58, 82). They may persist in a viable but noncultivable (VBNC) state on growth media, but maintain viability and pathogenicity, which can be restored in favourable environmental conditions (36, 37). This may also partly explain why the detection of *E. faecalis* using PCR method is much more sensitive than by culture. The detection limit of culture method is approximately 10^4 to 10^5 cells for target species using nonselective media, while for PCR method is 10 to 10^2 cells depending on the technique used (83, 84). PCR can detect free floating DNA, and DNA from nonviable and viable but noncultivable (VBNC) cells. In contrast, culture method requires viable and dividing cells (85). However, we examined only *E. faecalis* in endodontically treated teeth, meaning that they might have more microbial composition and diversity.

Only a small number of studies examined the relationships between the prevalence of *E. faecalis* and clinical parameters in root-filled teeth and the results are still inconclusive (11, 24-26). Kaufman and colleagues showed that the presence of periapical lesion significantly correlated with the presence of *Enterococcus* spp. (24) However, Zoletti and colleagues found that there was no significant difference between the occurrence of *E. faecalis* with and without periapical lesions (25). In addition, Wang and colleagues and Murad and colleagues found that the tooth locations, presence of clinical symptoms, type of coronal restoration, restoration status, number of root canals and quality of canal obturation were not significantly associated with the presence of *E. faecalis* in root canals. However, periapical lesions with the largest areas correlated with higher counts of gram-negative rod species (11, 26). Our results showed that the presence of signs and symptoms, presence of periapical lesion, size of periapical lesion, quality of previous root filling, and quality of the coronal restoration were not significantly associated with the presence of *E. faecalis* in root canals ($p > 0.05$). Due to small sample

size, we could not make a conclusion on the association between the prevalence of *E. faecalis* and clinical parameters and this needs further studies. However, the presence of *E. faecalis* in root canals mostly was isolated from a tooth with unacceptable of quality of previous root filling. Therefore the suitable root canal obturation, compacted void-free filling materials and at the correct length, may reduces the residual microorganisms. In addition, clinical parameters that may influence the prevalence of *E. faecalis* can influence endodontic success or failure of endodontic retreatment. Previous studies suggested that quality of the previous root filling, presence of a perforation, and apical periodontitis are significant outcome predictor of treatment success (50, 51).

E. faecalis can form dense aggregates or biofilm in root canals (2, 19, 43). Our results showed that different clinical isolates of *E. faecalis* showed different levels of biofilm formation. This may affect their ability to colonize in the root canals. In the root canal system, *E. faecalis* can produce several virulence factors that enhance adhesion, colonization and biofilm formation. The virulence genes of E. faecalis detected in teeth with failure of the endodontic treatment included *ace, efaA, esp, gelE, asa* and *asa373* (23) but the *ace* genes did not appear to involve in biofilm formation in primary infection (52). Several studies investigated the capacity of biofilm formation of *E. faecalis* clinical isolates. Anderson and colleagues investigated correlation between presence of virulence genes (*asa1, cylA,* and *esp*) and biofilm formation capacity of *E. faecalis* clinical isolates and found that root canal treated teeth had lower abundance of *cylA* and *esp* than all the other isolates. (53). Yoo and colleagues investigated correlation between biofilm formation and extracellular material (ECM) levels of *E. faecalis* clinical isolates. ECM surrounding *E. faecalis* might increase resistance to environmental stresses. (55). Bacteria in root canal treated teeth with apical periodontitis exist in the form of biofilm both in symptomatic and asymptomatic cases (57). In addition, biofilms are significantly associated with large epithelialized lesions (cysts and epithelialized granulomas or abscesses) but no significantly associated with clinical symptoms (56). Our results showed that different clinical isolates of *E. faecalis* had different biofilm formation capacity and this may affect their virulence. Interestingly, the clinical strain with the highest biofilm

mass was isolated from a tooth with intact coronal restoration but unacceptable of quality of previous root filling (void), while the other strains were from teeth with defective restorations. This may imply that the bacteria with high biofilm formation may have persisted from pretreatment. However, due to small sample size, we could not make a conclusion on the association between biofilm formation and clinical parameters and this needs further studies. In addition, molecular techniques have revealed the presence of other bacterial species in the root canals. In persistent extra-radicular infections, *Actinomyces israelii* and *Propionibacterium propionicum* was also prevalent (38). Using checkerboard DNA hybridization technique, mixed infection with *Enterococcus faecium* and *Streptococcus epidermidis* (11) Propionibacterium species, *Fusobacterium nucleatum*, Streptococci, and *Pseudoramibacter alactolyticus* is the most prevalent species (10). The other microorganisms are also found in root canal treated teeth such as *Fusobacterium nucleatum* and *Propionibacterium* spp.(86, 87). In recent years, Next-generation sequencing (NGS) has been used to assess bacteria diversity in root canals (39, 40). A systematic review of the root canal microbials reported that main species detected in root canal treated teeth are Fusobacterium, Actinomyces, Porphyromonas, Prevotella and Streptococcus (41, 42). Moreover, viruses and fungi have also been associated in endodontic disease (43). However, there is a significant difference in the prevalence of microorganisms between the geographical areas (42). Additional studies are required to determine the roles of these bacterial species in multispecies biofilm community.

In conclusion, *E. faecalis* was observed in approximately 76% of root canal treated teeth, using PCR analysis. Our result did not detect any *C. albicans* in the samples. No statistically significant association was observed between the prevalence of *E. faecalis* and any clinical parameters. Clinical strains of *E. faecalis* showed different levels of capability for biofilm formation.

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