# Quantitative Real-Time PCR of cps Type 1, 2 and 5 of Enterococcus faecalis and Candida albican Isolated from Infected Root Canal of Subject Requiring Endodontic Treatment

*Dewa Ayu N.P.A.[1]*, Boy M. Bachtiar[2], Siti M. Soerono Akbar[1], AndiArus Victor[3]*

1. Department of Dental Conservative, Faculty of Dentistry, Universitas Indonesia, Jakarta Indonesia.
2. Departemen and Laboratory of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta Indonesia.
3. Depatemen of Ophthalmology Faculty of Medicine, Universitas Indonesia.

## Abstract

Enterococcus faecalis and Candida albicans is a normal microflora in the oral cavity but can also be isolated from the root canal infection of primary and persistence cases.

Aim : The purpose of this research to investigate the amount of both E. faecalis and C. albicans and the capsular polysaccharide (cps) type isolated from root canal samples of Indonesian requiring endodontic treatment.

One hundred and fourteen outpatients attended three private hospitals in Jakarta Indonesia for endodontic treatment provided root canal samples. The clinical samples were collected from infected root canal teeth and grown on Chrom agar plate, while PCR was used to confirmed the species as well as their cps types. Additionally, the proportion of both E. faecalis and C. albicans in infected root canal was analyzed by using qPCR

The number of E. faecalis and C. albicans in endodontic retreatment group almost doubled compared to the group of primary endodontic treatment. Statistical test results showed a non-significant, P> 0.05. The correlation between the relative amounts of C. albicans and E. faecalis showed a strong linear relationship for groups of primary endodontic treatment with the value of 0.624 (p <0.001) as well as overall relations with the value of 0.514 (p <0.001). Endodontic retreatment group also has a strong enough relationship with a value of 0.399, p = 0.003. Overall cps 1 is more dominant (76,3,0%) compared to the combination of cps 1-2 (9,7%) and cps 1-5 (14%).

C. albicans and E. faecalis can be found as much as persistent microorganisms in the root canal endodontic retreatment cases. The average relative amount of E. faecalis in endodontic retreatment group was much higher while C. albicans is higher in primary groups. While the expression of capsular polysaccharide (cps) type 1, not encapsulated, dominates the encapsulated type cps 2 and 5.

***Experimental article (J Int Dent Med Res 2016; 9: (3), pp. 157-163)***

**Keywords:** E. faecalis, C. albican, cps 1, 2, 5, primary endodontic treatment and endodontic retreatment.

**Received date:** 26 June 2016 **Accept date:** 05 October 2016

## Introduction

*Enterococcus faecalis (E.faecalis)* and *Candida albicans (C. albicans)* are microflora normally seen but also the primary etiologies of the nasocomial infection. Both of them were also found in the oral cavity in root canal treatment failure cases.[1-4]

***Corresponding author:***
Dr. Dewa Ayu N.P.A., Department of Dental Conservative, Faculty of Dentistry, Universitas Indonesia, Jl. Salemba Raya No. 4 Jakarta 10430 Indonesia.
**E-mail:** dewaayunpa@yahoo.co.id

*E. faecalis* can also be isolated from root canal in teeth with pulpo periapical infection which isn't treated yet.[5-7] They are also the main etiology of the failed root canal treatment or Post Treatment Disease (PTD), because of their resistence from any treatment procedures and extreme condition, becoming persistence bacteria.[8-13]

*C. albicans* is the most pathogen fungi for human and the most four etiologies of nasocomial infection in vascular disease. *C. albicans* were also the most common fungi seen in oral cavity. [1, 2, 14]

The presence of *E. faecalis* and *C. albicans* in root canal have made them resistence pathogen. These microfloras formed biofilm making them hard to eliminate on primary endodontic treatment, becoming persistence and found in cases needed endodontic retreatment.[1, 2, 15-17]

Capsular polysaccharides expression has important role on virulence from many microorganisms. The presence of polysaccharide capsule made the microorganisms undetected from immune system.[18] Enterococci has polysaccharide capsule as an antigen for target host antibody increasing the pathogenic potential of *E.faecalis.* More understanding of this antigen would help in determining treatment choice of enterococci infection.[19]

Capsular expression can be used for knowing the genotype of *E. faecalis* with capsular typing. Capsular polysaccharides (cps) production has important role in the pathogensis to avoid immune responses. Recent studies have focused on detection *E.faecalis* mechanism in avoiding immune response making the infection still exist. Strain producing the capsule were proved to be more resistance than the strain with no capsule. [18, 20]

*Capsular polysaccharide* biosynthesis by *E.faecalis* were divided into 11 variety: cpsA-K. But only 7 of them producing the capsule, i.e: cpsC, cpsD, cpsE, cpsG, cpsI, cpsJ, cpsK. Genetically, *E. Faecalis* can be classified into 3 type according *capsule operon polymorphism*: cps 1, didn't express *capsular polysaccharide* while cps 2 and cps 5 expressed *Capsular polysaccharide*. cpa 1 presents cpsA and cpsB, cps 2 present all the genes of 11 cps and cps 5 brings together all genes except cpsF.[4, 18]

Detecting *E. faecalis* and *C. albicans* from clinical sample taken from the root canal can be done using culture or *real-time quantitative Polymerase Chain Reaction*(qPCR). qPCR was reported to be more sensitive than culture.[21, 22]

There are few researches focused on the difference amounof t*E. faecalis* dan *C. albicans* between primary endodontic treatment and endodontic retreatment cases along with the proportion of *capsular polysaccharide* (cps) in both cases. The purpose of this research was to investigated the differences of amount of both *E. faecalis* and *C. albicans* and the *capsular polysaccharide (cps*) type isolated from root canal sample Indonesian requiring primary endodontic treatment and endodontic retreatment. The method used in this research was qPCR.

## Materials and methods

### Experiment Materials

During the period of 12 months, samples were obtained from patients needed root canal treatment who came to specialistic clinic in three private hospitals in Jakarta, Indonesia. Sixty one needed primary endodontic treatment and 53 needed endodontic retreatment Patient informed consent was obtained. The mean age of the patient was 36.5 years (18-55 years). The patient must have good general health under no antibiotic prescription in the past month. Clinical findings shows intracanal infection symptoms and radiographic findings shows periapical radiolucency were included for primary endodontic treatment. The criteria for endodontic retreatment was failed root canal treatment cases.

### Clinical Sampling

Teeth that fall into the criteria for inclusion were isolated using rubber dam. The teeth surface were sterilized with$H_2O_2$ 30%, followed by NaOCl 2.5% and neutralized by sodium thiosulphat 5%.[21, 23]Access preparations were done using round bur #4, using #10 K-type file (Dentsply-Maillefer, Ballagues, Switzerland) to confirm patency and stopped 1mm before apical foramen.[24] Root canal preparations were done using #20 H-type file (Dentsply-Maillefer, Ballagues, Switzerland). Samples were taken by using sterile paper points into the root canal for 1 minute. Paper points were then transferred into Eppendorf tube containing 1 ml phosphate-buffered saline (PBS). The same treatment is done to the endodontically treated teeth, begin with removal the old restorations and root canal filling materials without using chemical solvent.

Samples in the Eppendorf tubes were homogenized and 10 uL aliquots were plated on chromogenic agar plates (Brilliance VRE; Oxoid, Basingstoke, UK) to get selective isolation of enterococci and incubated under temperature $37^0$C for 24 hours. The colony grown on chromogenic agar with bluish green color were suspected as *E.faecalis*. Then subculture were done to confirm by placing the colony on brain heart infusion (BHI, Oxoid) agar plates. Then left to stand for 24 hours (overnight) at a temperature of $37^o$C to be confirmed by PCR and will be used for further research.

**Real-time quantitative Polymerase Chain Reaction (RT q-PCR)**

DNAs were extracted from the samples in the Eppendorf tubes using DNA test tissue extraction kit (Real Biotech Co. USA) according manufacturer's guidance. Then, an amount of 1 mL sample was centrifugated under condition 5000 x g for 10 minutes.The supernatant was removed, then the pellets were resuspended with 180 µL nuclease-free water (Biosystems). digestion solution dan 20 µL Proteinase K. After that, samples heated at a temperature of 56 ° C for 30 minutes. Then, 20 µL RNAse A solution, 200 µL Lysis solution, and 400 µL etanol 50% were added and the solution was centrifugated again at 6000 x g for 1 minute. Then 500 uL Wash Buffer I was added and centrifuged at 8000 x g for 1 minute. After removing the supernatant, 500 uL Wash Buffer II was added and the solution was–centrifuged at full speed (≥12000 x g) for 3 minutes. Then 200 uL Elution Buffer was added to the sample and incubated at room temperature for 2 minutes. At last, the samples were centrifuged 8000 x g for 1 minute.Supernatant containing DNA ready to be used to continue the qPCR phase.

The primer pairs sequence for *E.faecalis* 16S rRNA gene were E16S 72f, 5' -CCGAGTGCTTGCACTCAATTGG-3'; E16S 210r, 5' -CTCTTATGCCATGCGGCATAAAC-3'.[22, 25] while the primer pairs sequence for *C.albicans* were PAN ACf 5' -TGGGTGGTAAAT TTCATCTAAAGCTA-3'; PAN ACr 5' -CAAGTKCTTTTCATCTTTCSWTCA-3'.Primer pairs used to detect cps 1, 2 dan 5 were : cpsB5-F 5' –CCAGGACAT GGTGGTATTTTAGATC-3' and Hcp1-R 5' -CGCCAATAAACAATCTTTACCAGAGC-3' producing amplification product 950 base pair (bp) *E. faecalis* CPS1. While primers cpsEend-F 5'–GAACCTACAACAATTA AAAAGC-3' and cps G-R 5'-GCATAGTATGTTAAGATTGATCC*A-3* were used to detec and quantify *E.faecalis* cps 2 dan 5 respectively.[4]

Based on previous experiments, qPCR assay containing *E.faecalis* or *C. albicans* as well as primer 16S rRNA in the set contains the components with a total volume of 10 uL reaction consists of: 5 µL SYBR Select Master Mix (containing SYBR GreenER dye, AmpliTaq DNA polymerase enzyme, Urasil-DNA glicosilase enzyme, ROX Passive Reference dye, dNTP mixture (dATP, dCTP, dGTP, dTTP, dan dUTP), and buffer), 0.5 uL x 100 pg/uL primer forward dan reverse each, 3 uLx 100 pg/uLDNA template (Biosystems). Nuclease free water (Biosystems) was added at amount of 1 µL so that the volume becomes 10 µL. Negative control qPCR (no DNA template) and negative qPCR amplification control (total DNA template *E.faecalis* or *C. albicans*) were duplicated along the samples throughout experiment. This is to normalize the background signals that may occur during the amplification process.[21, 22] qPCR amplification *E.faecalis* was performed in a thermocyler for 40 consecutive cycles of aninitial enzyme activator step at $95^0$C for 10 minutes, annealing step at $60^0$C for 1 minute. The same thing for *C. albicans* but there are differences in temperature and time with denaturation step at $95^0$C for 15 seconds, annealing step $54^0$C for 1 minute.[22, 23] The number of *C. albicans* or *E. faeclis* in clinical sample was quantify by using the formula has been described.[26]

**Statistical Analysis**

Comparison of the average amount of *E.faecalis* and *C. albicans* qPCR detection results in the group of primary endodontic treatment and endodontic retreatment was done using the Mann-Whitney test. While the average relative amount of *E.faecalis* and *C. albicans* in both groups was done using independent t-test. The correlation between the amount of *E.faecalis* and *C. albicans* in the group of primary endodontic treatment, endodontic retreatment were checked using Spearman correlation. Significance level was P<0,05.

The proportion of the combination of cps 1, 2 and 5 of primary endodontic treatment, endodontic retreatment and total groups was performed using chi square (p = 0.710).

**Results**

The average number of *C. albicans* in endodontic retreatment group almost doubled compared to the group of primary endodontic treatment. Statistical test results showed a non-significant, P> 0.05 (Table 1). The same pattern was seen in the number of *E. faecalis*. The average amount of *E.faecalis* in endodontic retreatment group almost doubled compared to the group of primary endodontic treatment. Statistical test results also showed a non-significant results,P > 0,05 (Table 1). Range of estimates of the average number of *C. albicans*

and *E. faecalis* at primary endodontic treatment groups came under the range estimated endodontic retreatment group, so that it becomes non-significant difference.

| | primary endodontic treatment (n=61) | | | endodontic retreatment (n=53) | | | p* |
|---|---|---|---|---|---|---|---|
| | Median | Mean | SD | Median | Mean | SD | |
| Amount of *C. Albicans* | 0,486 | 59,134 | 372,270 | 0,590 | 113,156 | 707,887 | 0,697 |
| Amount of *E. faecalis* | 90,974 | 208,876 | 355,513 | 105,307 | 424,351 | 1068,326 | 0,252 |

*Mann-Whitney test

**Tabel 1.** Difference of average number *C. Albicans* and *E. faecalis* in primary endodontic treatment and endodontic retreatment groups.

The average relative amount of *C. albicans* group of primary endodontic treatment is higher than in endodontic retreatment group. Nevertheless the results are not statistically significant, P> 0.05 (Table 2). While the average relative amount of *E. faecalis* in endodontic retreatment group higher than in the group of primary endodontic treatment. That difference was also not significant statistically, P>0.05 (Table 2).

| | primary endodontic treatment (n=61) | | | endodontic retreatment (n=53) | | | p* |
|---|---|---|---|---|---|---|---|
| | Median | Mean | SD | Median | Mean | SD | |
| Relative amount of *C. albican* | 0,00037 | 0,24434 | 1,46074 | 0,0082 | 0,06155 | 0,22664 | 0,297 |
| Relative amount 0f *E. faecalis* | 0,09731 | 0,53769 | 1,17171 | 0,13290 | 3,71449 | 14,42305 | 0,376 |

*independen t-test

**Tabel 2.** Difference of relative amount *C. Albicans* and *E. faecalis* in primary endodontic treatment and endodontic retreatment groups.

The range of estimates of the relative number of *C. albicans* in endodontic retreatment group came under the group of primary endodontic treatment. While the range of estimates of the relative amount of *E .faecalis* in primary endodontic treatment groups came under the endodontic retreatment group. It causes the differences were not statistically significant.

Correlation of the amount of *C. albicans* and *E. faecalis* did not show any significant relationship liner, P> 0.05. For groups of primary endodontic treatment value 0,137 (p = 0.295) and for a group of endodontic retreatment value 0.127 (p = 0.367). Likewise, as a whole did not show any correlation with the value of 0.001, p = 0.991 (Table 3).

The correlation between the relative amounts of *C. albicans* and *E. faecalis* showed a linear relationship to each group. For groups of primary endodontic treatment has a strong relation with the value of 0.624 (p <0.001) as well as overall relations with the value of 0.514 (p <0.001). Endodontic retreatment group also has a strong relationship with a value of 0.399, p = 0.003 (Table 3).

| *Correlation amount of C. Albicans and E. faecalis* * | primary endodontic treatment (n=61) | endodontic retreatment (n=53) | Total |
|---|---|---|---|
| Amount of C. Albicans and *E. faecalis* | 0,137 (p=0,295) | -0,127 (p=0,367) | 0,001 (p=0,991) |
| Relative amount of *C. albican* and *E. faecalis* | 0,624 (p<0,001) | 0,399 (p=0,003) | 0,514 (p<0,001) |

**Tabel 3.** Correlation of C. Albicans and E. faecalis in primary endodontic treatment and endodontic retreatment groups.

| Distribution of CPS 1, 2, & 5* | primary endodontic treatment (n=61) | endodontic retreatment (n=53) | Total |
|---|---|---|---|
| CPS-1 | 49 (80,3%) | 38 (71,7%) | 87 (76,3%) |
| CPS-1-2 | 5 (8,2%) | 6 (11,3%) | 11 (9,7%) |
| CPS-1-5 | 7 (11,5%) | 9 (17%) | 16 (14%) |
| Total | 61 (100,0%) | 53 (100,0%) | 114 (100,0%) |

* Chi square (p=0,710)

**Tabel 4.** Proportion of CPS 1, CPS 1-2, CPS 1-5 E. faecalis in primary endodontic treatment and endodontic retreatment groups.

There were no significant differences in the proportion of the combination of cps 1, 2 and 5 of primary endodontic treatment and endodontic retreatment groups. The proportion of cps 1 was not significantly different between the primary endodontic treatment group (83.6%) and endodontic retreatment group (88.7%). The proportion of the combination of cps 1, 2 in primary endodontic treatment group (8,2%) %) did not differ significantly compared to endodontic retreatment group (11,3%). The same result for the combination of cps 1, 5 in primary endodontic treatment group (11,5%) did not differ significantly with endodontic retreatment group (17%). See Table 4. Overall cps 1 is more dominant (76,3,0%) compared to the combination of cps 1-2 (9,7%) and cps 1-5 (14%).See Table 4.

## Discussion

Bacteria *E. faecalis* and *C. albicans* is a normal microflora in the oral cavity but can also be isolated from the root canal endodontic cases of primary and persistent.[5-13] *E. faecalis* and C.

albicans are two organisms that have the ability to survive in unfavorable conditions (starvation) so that ultimately can be found as a persistent microorganisms in the root canal. Siqueira et. al (2004), can detect 77% of *E. faecalis* and 9% of *C. albicans* in case of failure of endodontic root canal.[13] This study found the average amount of *E. faecalis* and *C. albicans* is almost twice as much in the case of endodontic retreatment, although the difference was not statistically significant. The possibilities are microorganisms in primary endodontic treatment surviving or penetrate into the root canal during or post-treatment endodontic.[27] The persistence of the microorganisms in the root canal because it is not eliminated at the time of instrumentation, and resistant to disinfection materials. In addition, these microorganisms enter the dentin tubules that are not untouchable with disinfection materials and mechanical cleaning and shaping.[27]

*E. faecalis* be persistent bacteria because it has a virulence factor that is capable of invasion into the dentinal tubule, resistant to unfavorable environmental conditions, are capable of forming biofilms, synthesize inductive stress proteins, generating a proton pump.[17] Meanwhile *C. albicans* can survive because it has the capacity of a single infectious agent, is able to survive in environments with minimal nutrition, bacterial co-aggregation, dimorphism, adapt to varying environmental conditions, adherence to tissue, producing hydrolytic enzymes, is able to form biofilms, modulation of the host immune response.[16] Characters owned by *E. faecalis* and *C. albicans* causes the microorganisms can survive so it can be found more in the case of endodontic retreatment compared primary endodontic cases, according to the results of this study.

This explanation can also be given in average yield relative amount of *E. faecalis* in endodontic retreatment group almost seven times higher than in the group of primary endodontic treatment. On the other hand, the average number of relatively *C. albicans* group of primary endodontic treatment is almost four times higher than in the group of endodontic retreatment. It shows that *C. albicans* is the most common fungal pathogen found in humans and a major cause of nasocomial infection. *C. albicans* is a dimorphic fungus found in the oral cavity as commensal microorganisms, can be a pathogen in case of changing environmental factors such as saliva, smoking status, oral topography and the status of oral microflora. Its growth also can be increased due to immune patients degradation and the use of antibiotics.[14, 28] *C. albicans* changed from commensal fungus that is commonly found in the oral cavity to be persistent in root canals in endodontic retreatment cases.

Although there are differences in the average number and average relative amount of *E. faecalis* and *C. albicans* in the group of primary endodontic treatment and endodontic retreatment, All the difference has had a statistically non significant. This happens because of the range of estimates of each group goes into the other groups. The reason may be because there is a small value in one group or the amount of *E. faecalis* and *C. albicans* is almost the same.

It was explained that one of the characters of *E .faecalis* and *C. albicans* is the ability to form biofilms.[16, 17]It answers the finding that there is no correlation between the number of *C. albicans* and *E. faecalis* in primary endodontic treatment group or a group of endodontic retreatment. In a separate conditions, these microorganisms are not related. On the other hand, there is a correlation between the relative amounts of *C. albicans* and *E .faecalis* in both groups. Primary endodontic treatment group has a stronger relations, while endodontic retreatment group has a strong enough relationss. This means that they correlate when compared with other microorganisms isolated from root canals. The cause of endodontic infections are poly microbes and fungi are very common detected in root canal from pulpal disease.[29] Previous studies have found that *C. albicans* and *E. faecalis* simultaneously detected on the isolation of primary cases endodontic root canal and endodontic retreatment even in the different number and percentage. Both persistent microorganisms are widely researched to find the best way to eliminate them.[2, 13-15, 30, 31] Lima et.al (2015) found that *C. albicans* and *E. faecalis* contribute to prainflamatory and antimicrobial response.[15] As for how the cooperation of both to become increasingly resistant, require further study so the efforts to destroy these persistent microorganisms can be optimized.

*E. faecalis* type of cps 1 (83.6%) is almost always found from the isolated root canal,

because the bacteria with the type of cps 1 is a bacterium that is not encapsulated. These results are consistent with the results of qPCR which found *E. faecalis* in all root canal study subjects both in primary endodontic treatment and endodontic retreatment. The proportion of the combination of cps 1, 2 and cps 1, 5 in the two groups more in number in endodontic retreatment group although it was not significantly different. In other words, type 2 cps more easily detected after endodontic treatment. Both of them are relatively constant. Although quantitatively less, further investigation will be needed whether this type of cps 2 is more virulent.

Capsular polysaccharides play an important role as a virulence factor of a microorganism. The function of capsules in *E. faecalis* is blocking pathogen detection surface resulting in decreased production of cytokines by macrophages.[4] Their capsules also have important role for microbes to escape detection by the host immune system.[18] Although the majority of root canal isolation results from this study that *E .faecalis* dominated by cps 1 which is the type that is not encapsulated, but also cps types 2 and 5, which are capsulated, are proved to be detected. Pinheiro et.al (2012) found that although the percentage of cps 2 discovery is relatively small (16.7%) but these strains resistant to antibiotics compared to other strains.[4]

That finding agrees with the results of this research. Scarce detection of cps 2 may be because not all strains of cps 2 express its capsule because an insert models, adding strength to the cell adhesion and increase biofilm formation.[32] Further researches are still needed to find out how the virulence factors contribute to these three cps types on endodontic therapy and become persistent bacteria.

## Conclusions

It is concluded that *C. albicans* and *E. faecalis* can be found as much as persistent microorganisms in the root canal endodontic retreatment cases. It can survive because it has virulence factors. The average relative amount of *E.faecalis* in endodontic retreatment group was much higher while *C. albicans* is higher in primary groups. While the expression of capsular polysaccharide (cps) type 1, not encapsulated, dominates the encapsulated type cps 2 and 5. Although *C. albicans* and *E. faecalis* is almost always found together in the root canal, the relation of both to become the most persistent microorganisms is still not known clearly and require further researches. The role of cps in increasing the virulence of *E. faecalis* still require more research, especially its resistance to a variety of materials and tools endodontic.

## Acknowledgements

This study was performed using personal funds. This research is a preliminary study of the doctoral program at Department of Dental Conservative, Faculty of Dentistry, Universitas Indonesia. The author wish to thank Prof. Boy M. Bachtiar, drg., MS., Ph.D and Prof. Dr. S.M. Soerono Akbar, drg., SpKG(K) for their suggestions and supports during the research.

## Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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