Evaluation of Antimicrobial Efficacy of Antibiotics and Calcium Hydroxide against *Enterococcus faecalis* Biofilm in Dentine

(Kajian Keberkesanan Antibiotik Antimikrob dan Kalsium Hidroksida ke Atas Biofilem *Enterococcus faecalis* di Dalam Dentin)

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ABSTRACT

The objective of this study was to investigate the antimicrobial efficacy of erythromycin, oxytetracycline and calcium hydroxide [Ca(OH)$_2$] against Enterococcus faecalis *biofilm in dentine*. E. faecalis ATCC 29212 *(American type culture collection) was inoculated into standard tooth sections and incubated in aerobic atmosphere at 37°C for 21 days. The infected tooth sections were then exposed to the test agents for 5 and 10 min. The colony forming units (CFU) after the exposure periods at three different depths <100 µm, 100-350 µm and 350-500 µm were enumerated. After 5 min of exposure, both antibiotics had significantly lower CFU count than Ca(OH)$_2$ solution at three dentinal depths. Comparing with the oxytetracycline, the CFU count of the erythromycin was significantly (p<0.05) lower at the depth of 100-500 µm. Similarly, after 10 min of exposure, erythromycin had significantly lower CFU count (p<0.05) at three dentinal depths. Oxytetracycline showed significantly lower CFU count than Ca(OH)$_2$ at 100 µm depth. Comparing with the two exposure times, the erythromycin and Ca(OH)$_2$ groups showed significant lower CFU counts after 10 min of exposure in the antimicrobial agents to 5 min. In conclusion, both antibiotics show better antimicrobial activity than Ca(OH)$_2$ in removing the* E. faecalis *biofilm in dentine.*

Keywords: Antimicrobials; biofilm; calcium hydroxide; dentinal tubules; Enterococcus faecalis

ABSTRAK

Tujuan kajian ini adalah untuk mengkaji keberkesanan antimikrob eritromisin, okstetrasilisin dan kalsium hidroksida [Ca(OH)$_2$] ke atas biofilem Enterococcus faecalis *di dalam dentin*. E. faecalis ATCC 29212 *(American Type Culture Collection) diguna untuk menjangkiti keratan piawai akar gigi manusia dalam keadaan atmosfera aerobik pada 37°C selama 21 hari. Keratan akar gigi yang telah dijangkiti didedahkan kepada ejen ujian selama 5 dan 10 min. Selepas tempoh pendedahan, kiraan unit pembentukan koloni (CFU) pada tiga kedalaman dentin, iaitu <100 µm, 100-350 µm dan 350-500 µm yang berbeza dibandingkan. Selepas pendedahan selama 5 min, kedua-dua jenis antibiotik didapati mempunyai kiraan CFU yang kurang namun signifikan berbanding Ca(OH)$_2$ pada ketiga-tiga kedalaman dentin. Berbanding dengan okstetrasilisin, eritromisin menunjukkan kiraan CFU yang jauh lebih kurang berbanding okstetrasilisin dan signifikan (p<0.05) pada kedalaman dentin 100-500 µm. Selepas pendedahan selama 10 min, eritromisin mempunyai kiraan CFU yang paling rendah dan signifikan (p<0.05) pada ketiga-tiga kedalaman dentin. Okstetrasilisin menunjukkan kiraan CFU yang ketara kurang daripada Ca(OH)$_2$ pada kedalaman 100 µm. Dalam perbandingan masa pendedahan, eritromisin dan Ca(OH)$_2$ menunjukkan CFU yang ketara kurang selepas pendedahan selama 10 min berbanding dengan 5 min. Secara kesimpulan, kedua-dua antibiotik menunjukkan aktiviti antimikrob yang lebih berkesan daripada Ca(OH)$_2$ terhadap biofilem* E. faecalis *pada dentin.*

Kata kunci: Antimikrob; biofilem; kalsium hidroksida; Enterococcus faecalis*; tubul dentin*

INTRODUCTION

Enterococcus faecalis (*E. faecalis*), a facultative anaerobe, has been commonly isolated from persistent root canal infections and post-treatment infection (Molander et al. 1998; Sundqvist et al. 1998). One of the virulence factors associated with this persistent presence of *E. faecalis* in root canals is its ability to invade dentinal tubules, adhere to collagen in the dentine (Love 2001). An *in-vitro* study has shown that albeit with the presence of intracanal medications, the *E. faecalis* was able to form biofilm in the root canals (Distel et al. 2002). Biofilm is a highly organized structure with clumps of bacteria bound together by a carbohydrate matrix (Costerton et al. 1994). In addition, the *E. faecalis* has the ability to survive without dividing in the biofilm (Distel et al. 2002) and induced the apatite reprecipitation, especially in a mature biofilm (Kishen et al. 2006). Hence, these features explain the low susceptibility of *E. faecalis* biofilm towards several antimicrobial agents when compared to its other morphotypes such as the planktonic and pellet forms (Abdullah et al. 2005). The

polysaccharide coating of the biofilm could be the barrier to most antimicrobial agents (Distel et al. 2002).

Several studies have shown that the *E. faecalis* is highly resistant to the antibacterial effect of calcium hydroxide [Ca(OH)$_2$] (Haapasalo & Orstavik 1987; Orstavik & Haapasalo 1990; Siqueira & de Uzeda 1996). Evans et al. (2002) reported that *E. faecalis* can survive in an alkaline environment as high as pH11.1 and could only be killed when pH reached higher than 11.5. However, the pH of the Ca(OH)$_2$ in the canal could only reached up to 10.3 due to the buffering effect of the radicular dentin (Evans et al. 2002).

Knowing the limitation of the standard intracanal medication [Ca(OH)$_2$], various methods such as the use of laser (Mehrvarzfar et al. 2011; Sahar-Helft et al. 2011), ultrasonic (Grundling et al. 2011), different instrumentation systems (Gorduysus et al. 2011; Matos Neto et al. 2011) for the eradication of *E. faecalis* have been reported. As most root canal infections are of bacterial origin, it is not surprised that there were new antimicrobial agents containing antibiotic such as the MTAD, a mixture of tetracycline isomer, an acid and a detergent (Tong et al. 2011; Torabinejad et al. 2003) and Tetraclean, a mixture of doxycycline, citric acid and polypropylenglycole (Giardino et al. 2006; Neglia et al. 2008) have been developed. The presence of antibiotics in these new generations of antimicrobial agents suggests that the antibiotics may play an important role in the root canal therapy.

Several studies have investigated the efficacy of various types of antibiotics such as clindamycin (Gilad et al. 1999; Lima et al. 2001; Lin et al. 2003; Molander et al. 1990), erythromycin (Molander & Dahlen 2003), metronidazole (Lima et al. 2001), tetracycline (Giardino et al. 2006; Lin et al. 2003; Molander & Dahlen 2003; Tong et al. 2011; Torabinejad et al. 2003) and a mixture of ciprofloxacin, metronidazole and minocycline (Hoshino et al. 1996). In addition, the application of antibiotics in different forms, such as paste (Hoshino et al. 1996; Lin et al. 2003; Molander & Dahlen 2003; Molander et al. 1990; Siqueira & de Uzeda 1996), gel (Lima et al. 2001), solution (Giardino et al. 2006; Neglia et al. 2008; Tong et al. 2011; Torabinejad et al. 2003) or impregnated in a delivery vehicle (Gilad et al. 1999) have been investigated. It was suggested that when the antibiotics were applied locally into the infected root canals instead of administered systematically, it would reduce the risk of adverse systemic effects (Mohammadi 2009). The aim of this study was to evaluate the antimicrobial effectiveness of local application of two antibiotics and Ca(OH)$_2$ against *E. faecalis* biofilm in dentin.

MATERIALS AND METHODS

The method of this study was modified from a previous study (Haapasalo & Orstavik 1987). Extracted human anterior teeth (excluding lower incisors) were disinfected overnight in 0.5% sodium hypochlorite (NaOCl). The apical 5 mm of the roots was removed using a rotating diamond saw at 1000 rpm (Isomet® 1000 Precision Sectioning Saw, Buehler Ltd., USA) under water cooling (Coolmet™, Buehler Ltd., USA). Subsequently, the roots were cut transversely into slices of 4 mm thick. The internal diameter of the root canal was shaped to a standardized size of 1.4 mm with an ISO size 014 (No. 4) round steel bur (ASH, Amalgamated Dental Trade Distributors Ltd, England) run in a slow-speed contra-angle handpiece. The cementum layer of the tooth sections remained intact.

The smear layer of the root canal was removed by treating the tooth sections in an ultrasonic bath of 17% EDTA (Ethylenediaminetetraacetic acid disodium salt, R & M Marketing, UK) (pH7.8) followed by 5.25% NaOCl for 4 min each. The tooth sections immersed in brain-heart infusion broth (BHI) (BHI, Scharlau Chemie SA, Spain) were then sterilized in an autoclave at 121°C for 20 min. The sterility of the tooth sections was checked by observing any changes of the cloudiness of the broth solution after a 24 h of incubation in an aerobic incubator.

The sterile tooth sections were then inoculated with 1×10^8 CFU/mL *E. faecalis* ATCC 29212 (American type culture collection) and incubated under aerobic condition at 37°C for 21 days. At the end of the 21 days of incubation period, the tooth sections were washed 3 times with fresh sterile PBS to remove excess medium and planktonic form of bacteria. The infected tooth sections were divided into 6 groups with 12 tooth sections in each group. They were exposed to 10 mL of the following test agents and incubated in an aerobic incubator at 37°C: Group 1 & 2 tooth sections were exposed to calcium hydroxide solution [Ca(OH)$_2$] (Calcium hydroxide p.a., Merck, Germany) at pH12.3 for 5 and 10 min, respectively; group 3 & 4 tooth sections were exposed to erythromycin (Erythromycin Lactobionate IV, Abbott Laboratories, USA) for 5 and 10 min, respectively. The erythromycin solution was prepared by mixing the 500 mg erythromycin powder with 10 mL of water for injection and Group 5 & 6 tooth sections were exposed to oxytetracyline (Oxylim, Atlantic Laboratories Corp Ltd., Thailand) for 5 and 10 min, respectively. The oxytetracyline was available as 500 mg in 10 mL solution form. In each group, two tooth sections were used as a control, in which they were exposed to sterile PBS for 5 and 10 min, respectively.

At the end of each exposure time, the antimicrobial activities of the test agents were ceased by washing the tooth sections with 10 mL of sterile PBS for 5 times. The dentine samples of the inner surface of the tooth sections were collected using three different diameters of sterile steel round burs [ISO sizes 016 (#5), 021 (#7), 023 (#8)] run in a slow speed handpiece. These burs excavated the dentine samples at <100 μm, 100-350 μm and 350-500 μm depth of root dentine, respectively. After each drilling, the dentine powder on the surfaces of the tooth sections and respective burs was collected by rinsing with 5 mL of BHI broth into a sterile bottle. The bacterial were serially diluted and inoculated on Columbia horse blood agar plates (Biomedia, Utas Maju Sdn. Bhd., Malaysia) and

incubated in aerobic atmosphere at 37°C. The colony-forming unit per tooth section were enumerated after the 24 h of incubation period.

To determine the formation of biofilm, two infected tooth sections were prepared and examined under scanning electron microscope (SEM). Briefly, at the end of the 21days incubation, the tooth sections were fixed in 4% glutaraldehyde 0.1 M cacodylate buffer solution (Agar Scientific Ltd., United Kingdom) and subsequently post-fixed for 2 h in 2% osmium tetroxide (Agar Scientific Ltd., United Kingdom). Dehydration was performed in a series of ascending ethanol concentration (30%, 50%, 70%, 95% and 100%) for 15 min each. The tooth sections were then further dried in a critical point dryer (Polaron CPD7501, Quorum Technology Ltd., United Kingdom). Following that, the tooth sections were split into two and sputter coated with a layer of 200Å gold palladium (BIO-RAD, Bio-rad Microscience Division, United Kingdom) before examination under SEM (Philips SEM 515, Philips Industrial & Electro-acoustic Systems, Netherlands).

The data were entered into a Statistical Package for Social Science (SPSS) software (Version 11.0; SPSS Inc, Chicago, IL, USA). One way ANOVA was used to compare the CFU counts of the test groups. Independent t-test was used to analyse the difference of CFU counts at the two exposure times. A p-value of <0.05 was regarded as statistically significant.

RESULTS

There was evidence of colonization of *E. faecalis* biofilm on the tooth sections under SEM examination (Figure 1). The bacterial colonies encompassed in a dense matrix, appeared as a mushroom-shaped colonies on the root canal surface were identified.

An overall comparison of the antimicrobial efficacy of the three test agents after 5 and 10 min of exposure time were shown in Figures 2 and 3, respectively.

After 5 min of exposure time, the $Ca(OH)_2$ has the significant ($p<0.05$) highest remaining viable bacterial count at the three dentine depths compared with the erythromycin and oxytetracyline groups (Figure 2). This suggests that the $Ca(OH)_2$ was the least effective antimicrobial activity against the *E. faecalis* biofilm on the tooth sections compared with the antibiotic groups. In contrast, the erythromycin showed a better antimicrobial activity. It had the lowest bacterial counts and was significantly lower compared with the other 2 test agents in the dentine depth of 100-500 μm (burs #7 and #8) (Figure 2).

Similarly, after 10 min of exposure, the highest and lowest remaining viable bacterial counts were the $Ca(OH)_2$ and erythromycin groups, respectively (Figure 3). The CFU counts of $Ca(OH)_2$ was significantly higher than the erythromycin group at all three dentine depths ($p<0.05$) but was not statistically different when
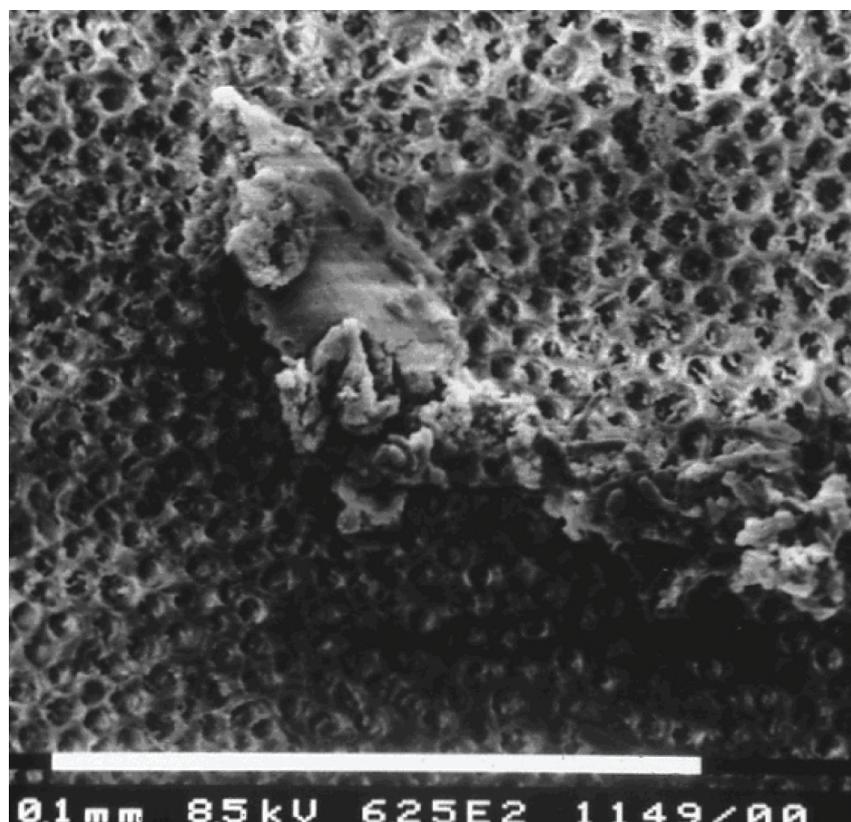

FIGURE 1. A SEM view of the root canal surface in an infected tooth section. Note a mushroom-shaped colony of *E. faecalis* embedded in polysaccharide matrix and penetration of bacterial colony into dentinal tubule (Scale bar = 0.1 mm)
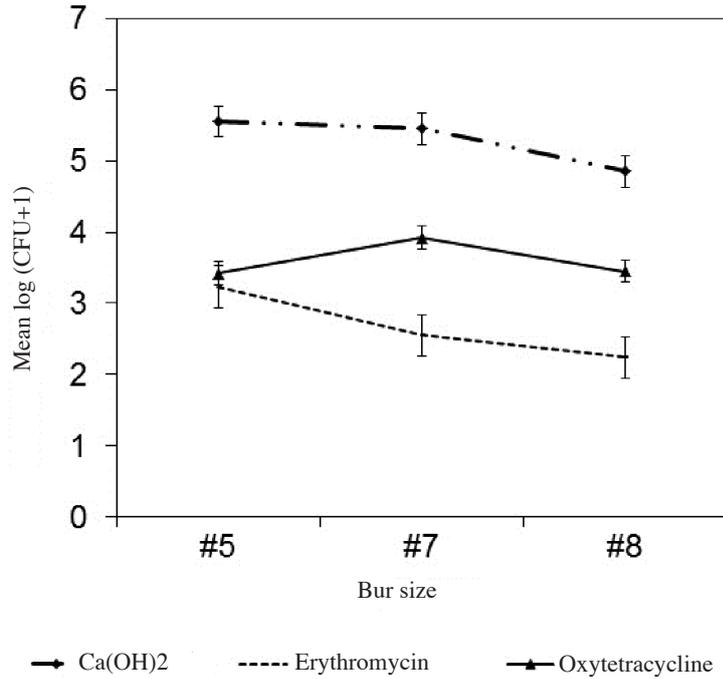
FIGURE 2. Mean $\log_{10}$ (CFU+1) of *E. faecalis* at three dentine depths after 5 min of exposure time to three test agents
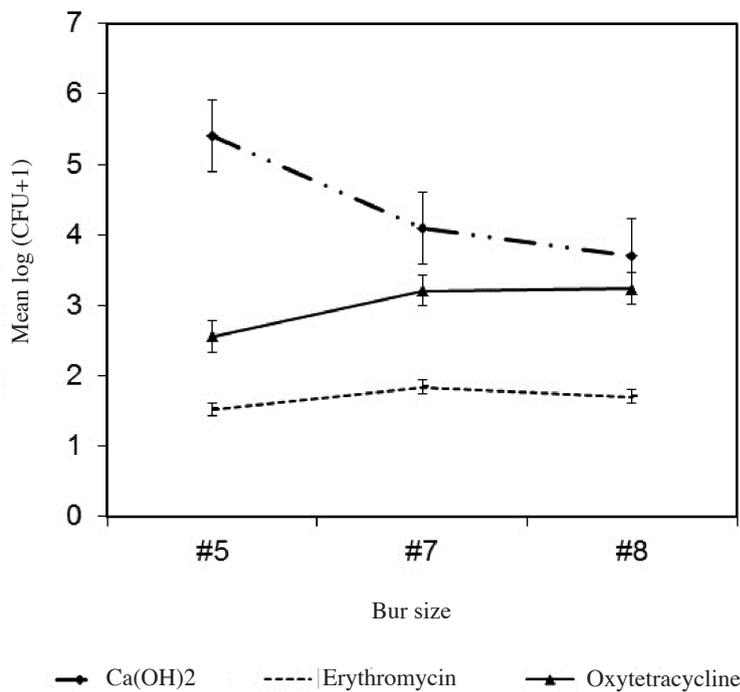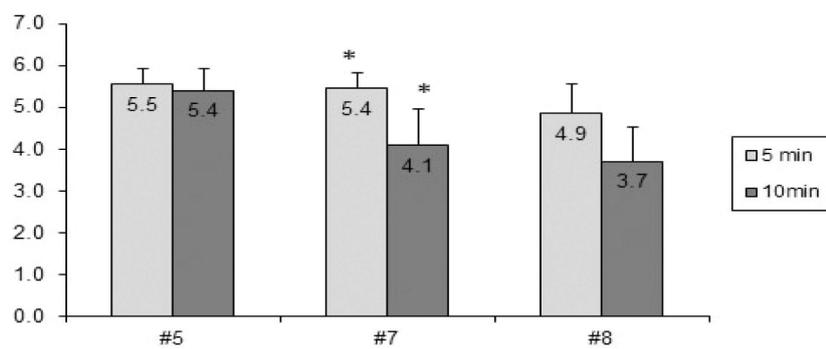

FIGURE 3. Mean $\log_{10}$ (CFU+1) of *E. faecalis* at three dentine depths after 10 min of exposure time to three test agents

compared with the oxytetracycline in deeper layers (burs #7 & #8).

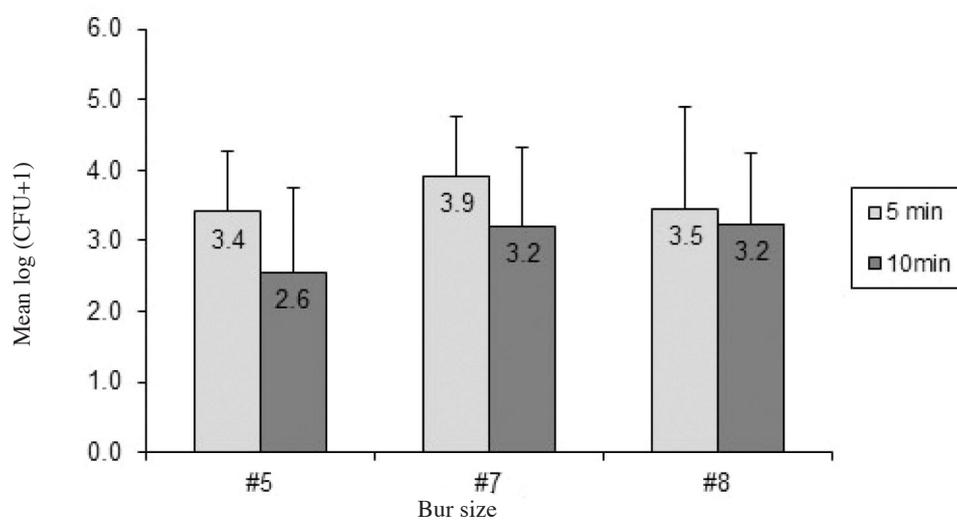In general, the CFU count was lower after a longer exposure period to the test agents (Figure 4). There were statistically lower CFU counts after 10 min of exposure in $Ca(OH)_2$ (Figure 4(a)) and erythromycin (Figure 4(b)) groups, at samples taken with burs #7 and #5, respectively. No statistically different of CFU count in

(a)

(b)

Mean log (CFU+1)

Bur size
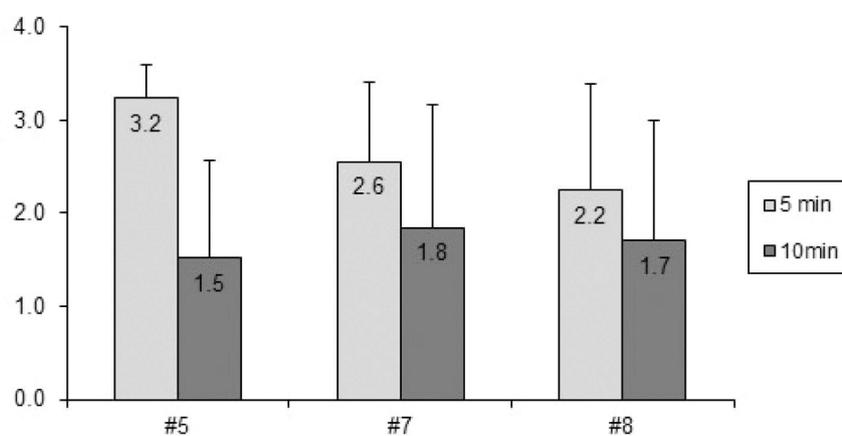
(c)

FIGURE 4. Comparison of the mean $\log_{10}$ (CFU+1) of *E. faecalis* at 5 and 10 min of exposure to (a) Ca(OH)$_2$, (b) erythromycin and (c) oxytetracycline [* $p<0.05$]

oxytetracyline (Figure 4(c)) group after the 2 exposure times.

Discussion

In this study, the infected tooth section model which was introduced by Haapasalo and Ørstavik (1987) was modified for the investigation of the antimicrobial efficacy of two antibiotics and Ca(OH)$_2$ against the *E. faecalis* biofilm in dentinal tubules. This model provides a standard height and internal diameter of the tooth sections, therefore reducing the variation of root canal morphology that is normally seen in full root length of extracted teeth. The cementum layer of extracted human anterior teeth was not removed in this study, so as to simulate the clinical conditions.

Many previous studies undertaken using Haapasalo and Ørstavik's model had incubated dentinal blocks for 3 weeks (Almyroudi et al. 2002; Lin et al. 2003; Safavi et al. 1990; Siqueira & de Uzeda 1996;) but none had directly used the term 'biofilm' when analyzing their results. The non-existence of term 'biofilm' is inevitable, as the use of this terminology in endodontology was not widely appreciated until Sen et al. (1999) made a reference to it. In our study, the 3 weeks incubation period was sufficient for the formation of clumps of bacteria bounded by a carbohydrate matrix, which was a feature normally found in 'biofilm'. This finding was in accordance with other studies, which also observed biofilm formation on dentine after a similar incubation period (Baca et al. 2011; George et al. 2005; Kishen et al. 2006). In fact, another study has reported that in a starvation condition, the *E. faecalis* biofilm could develop as early as after 1 day of incubation period.

In our previous study (Chai et al. 2007), by using an *E. faecalis* biofilm on membrane filter model, we evaluated the antimicrobial efficacy of a few antibiotics (ampicillin, co-trimoxazole, erythromycin, oxytetracycline, vancomycin and vancomycin followed by gentamicin) and Ca(OH)$_2$. These antibiotics are available in pharmacy for parenteral administration purpose. There were all in solution form, which can easily be injected into the root canals. Thus, we are interested to evaluate the antimicrobial effect of these commercially available antibiotics for the local application into the root canals. The previous results showed that the erythromycin, oxytetracycline and Ca(OH)$_2$ were able to produce a total eradication of *E. faecalis* biofilm after 1 h of exposure (Chai et al. 2007). Hence, these three agents were selected to be tested in infected tooth section model in this study. In order to determine the optimum exposure time for these three antimicrobial agents, a serial pilot study was carried out on the membrane filter model as well as the infected tooth section model. Based on the results of the pilot study, 5 and 10 min of exposure time were tested in the current study.

It was found that the commonly used intracanal medication of Ca(OH)$_2$ was not effective in killing *E. faecalis* biofilm in dentinal tubules after 5 and 10 min of exposure in this study. This finding was in accordance with other researchers' findings that reported the different exposure times of Ca(OH)$_2$ which ranged from 1 min to 30 days (Gomes et al. 2003; Haapasalo & Orstavik 1987; Safavi et al. 1990; Siqueira & de Uzeda 1996). However, this was in contrast with the finding of Almyroudi et al. (2002) who reported that Ca(OH)$_2$ was effective against dentinal tubules infected with *E. faecalis* after a 3 to 8-day exposure. The differences could be due to the different forms of Ca(OH)$_2$ used such as paste or viscous vehicle and the duration of the infected dentine.

It is known that the antibacterial activity of Ca(OH)$_2$ depends on its high pH. The alkalinity of this agent destroys bacterial cell membranes and protein structures (Siqueira & de Uzeda 1996). However, the initial high pH of Ca(OH)$_2$ at 12.3 would reduced to a pH of 10.3 when it was placed into the root canals (Evans et al. 2002). This pH reduction is due to the buffering effect of radicular dentine (Haapasalo et al. 2000; Nerwich et al. 1993; Siqueira & de Uzeda 1996). It has been known that the *E. faecalis* can survive at a pH as high as 11.5 (Evans et al. 2002), hence, with the lower pH value of Ca(OH)$_2$, the *E. faecalis* in the dentinal tubules could not be removed effectively. The buffering effect of the radicular dentine explained well why the Ca(OH)$_2$ showed total eradication of *E. faecalis* biofilm in membrane filter model (Chai et al. 2007) but not on infected tooth section models as in this study.

This study showed that both antibiotics were found to be more effective than the Ca(OH)$_2$ in the infected tooth section models. This finding was in accordance with an *in-vitro* study which revealed that the amoxicillin+clavulanate, ciprofloxacin, clindamycin and doxycycline were significantly more effective than Ca(OH)$_2$ in against the *E. faecalis* biofilm in root canal system (Saber Sel & El-Hady 2012). In an *in-vivo* study, it was reported that the enterococci isolated from persistent infected root canals treated with Ca(OH)$_2$ was not susceptible to tetracycline but was sensitive to erythromycin (Dahlen et al. 2000). In our results, the erythromycin showed a better antimicrobial effect when compared with the oxytetracycline group.

Although the erythromycin showed the most effective antimicrobial activity among the 3 test agents, it was still not able to completely eradicate the *E. faecalis* biofilm in dentinal tubules after both the exposure times. This could be attributed to the inability of these agents to penetrate into the dentinal tubules, inadequate exposure times (despite the results obtained during a pilot study indicating otherwise) or the concentration of antibiotic used. As some antibiotics can only exert their antimicrobial effects against metabolically active bacteria, they may need longer exposure time to be effective. In addition, higher antibiotic concentration is needed to eradicate the biofilm phenotype compared with their counterpart planktonic form (Sandoe et al. 2006). Thus, the optimal exposure times and concentration for antibiotics groups need to be further investigated. The present study indicated that the usage of antibiotic can be effectively confined within the root canal space, hence avoiding the dreadful need to administer it systemically, which carries a higher risk for

the development of drug-resistant bacteria. Within the limitation of this study, the antimicrobial efficacy of both the antibiotics was shown to be more effective than the Ca(OH)$_2$, but none were able to completely eradicate *E. faecalis* biofilm in dentinal tubules.

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