**CLINICAL EFFICIENCY OF 2% CHLORHEXIDINE GEL IN REDUCING
INTRACANAL BACTERIA**

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ABSTRACT

CHING S. WANG: Clinical Efficiency of 2% Chlorhexidine Gel in Reducing Intracanal Bacteria.

(Under the direction of Dr. Fabricio B. Teixeira)

This study evaluated the clinical efficacy of 2% chlorhexidine (CHX) gel on intracanal bacteria reduction during root canal instrumentation. The additional antibacterial effect of an intracanal dressing ($\text{Ca}(\text{OH})_2$ mixed with 2% CHX gel) was also assessed. Forty-three patients with apical periodontitis were recruited. Teeth were instrumented using rotary instruments and 2% CHX gel as the disinfectant solution. Bacterial samples were taken upon access (S1), after instrumentation (S2), and following 2 weeks of intracanal dressing (S3). Anaerobic culture was performed. Of the samples cultured positively at S1, 10.3% (4/39) and 8.3% (4/36) sampled bacteria at S2 and S3, respectively. A significant difference in the percentage of positive culture between S1 and S2 ($p < 0.001$), but not between S2 and S3 ($p = 0.692$), was found. These results suggest that 2% CHX gel is an effective root canal disinfectant and additional intracanal dressing did not significantly improve the bacteria reduction on the sampled root canals.

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ABBREVIATIONS

Ca(OH) ₂	Calcium Hydroxide
CHX	Chlorhexidine
CFU	Colony Forming Unit
EDTA	Disodium ethylenediamine-tetraacetate
NaOCl	Sodium Hypochlorite
NiTi	Nickel-Titanium
SS	Stainless-Steel
RPM	Revolutions Per Minute
LDTM	Liquid Dental Transport Medium

CHAPTER 1

INTRODUCTION

1a. Apical Periodontitis

Apical periodontitis is an inflammatory process on the periradicular tissues caused by the microbes presented inside root canal system (Kakehasi *et al.* 1965, Sundqvist 1976, Möller *et al.* 1981, Fabricius *et al.* 1982a). It is essentially the body's defense response to the damage of the pulpal tissue and microbial infection of the root canal system.

Research in the past three decades has established the essential role of microorganisms in the pathogenesis of apical periodontitis. As early as in later 1890, Miller demonstrated the presence of bacteria in necrotic pulp. Later, Kakehasi *et al.* (1965) showed that no apical periodontitis developed in germ-free rats when their molar-pulps were kept exposed to the oral cavity, as compared with control rats with a conventional oral microflora in which massive periapical radiolucencies occurred. Möller *et al.* (1981) demonstrated that the bacteria, not the necrotic pulp tissue, was the etiology for periapical inflammation in his monkey study. Sundqvist (1976) investigated previously traumatized teeth with necrotic pulp. The study showed that 18 out of 19 teeth with radiographic sign of apical periodontitis had cultivable bacteria; whereas, no bacteria could be isolated from the root canal of teeth without apical periodontitis. Bacterial species were predominately obligate anaerobes including *Fusobacterium*, *Porphyromonas* (formerly *Bacteroides*), *Prevotella* (formerly

Bacteroides), *Eubacterium*, *Peptococcus*, *Peptostreptococcus* and *Camylobacter*.

Baumgartner and Falkler (1991) demonstrated that teeth with carious pulpal exposures and periapical lesions were predominately colonized by anaerobic bacteria in the apical 5mm.

The endodontic environment provides a selective environment for establishment of a mixed root canal flora. It is predominated by Gram-negative anaerobic bacteria, due to the lack of oxygen and the availability of host tissues and tissue fluids as the primary nutrient source (Sundqvist *et al.* 1992). In a monkey study, Fabricius *et al.* (1982a) inoculated indigenous oral flora into root canal system and sealed the canals for a period of 1080 days. In the early stage, the root canal flora was predominately colonized by facultative bacteria. Shortly, strict anaerobes displaced the facultative bacteria. After 1080 days, 98% of bacteria isolated from the root canal system were strict anaerobes. In another study, Fabricius *et al.* (1982b) showed the mixed infections had the greatest capacity to induce apical periodontitis. He studied the ability of 11 bacterial strains, in various combinations, to induce periapical reaction in a monkey model. Other than *Enterococcus*, all other strains cannot survive as pure culture in the root canal system. The facultative anaerobe identified in most samples can only induce weak periapical reaction, while the combination of facultative and strict anaerobes can induce profound periapical inflammation. Based on those studies, it appeared there is selective pressure by the endodontic environment that favors the survival of strict anaerobes. In addition, certain combinations of bacteria are required to induce apical periodontitis, suggesting positive, mutually supportive, as well as negative interactions between bacterial species.

Bacteria can potentially penetrate dentinal tubules and protect themselves from the mechanical and chemical destruction during the root canal therapy. Ando and Hoshino

(1990) demonstrated the presence of bacteria 500 to 2000µm in dentinal tubules in carious human teeth by light microscopy. Love *et al.* (1996) showed bacteria invaded and penetrated deeper in cervical and mid root than the apical root. In some case, bacteria can potentially penetrate nearly to the cementum layer of the roots in teeth with apical periodontitis (Peters *et al.* 2001). Matsuo *et al.* (2003) also demonstrated that 70% of tubules had bacteria penetrated up to cementum using immunohistochemistry methods.

Bacterial species found in the dentinal tubules are similar to those found in the main root canal system. Ando and Hoshino (1990) showed the predominate bacteria found in the dentinal tubules was *Lactobacillus*, *Streptococcus*, *Propionibacterium*, and *Peptostreptococcus*. Matsuo *et al.* (2003) showed the presence of *Fusobacterium*, *Eubacterium*, *Lactobacillus* and *Peptostreptococcus* in the dentinal tubules.

Due to the anatomic restriction, bacteria in the tubules are difficult to remove by instrumentation and irrigation. Even with intracanal medications, viable bacteria were found in infected human dentin tubules after the treatment (Weiger *et al.* 2002). Although the clinical significance of bacteria in dentinal tubules is still not clear, studies tend to agree that the remaining bacteria in the tubules after the root canal treatment can contribute to failure of endodontic therapy (Love and Jenkinson 2002, Oguntebi *et al.* 1994, Peters *et al.* 2001).

In addition to bacteria, fungi and viruses were also detected in the infected root canal system. In teeth with apical periodontitis, the presence of fungi has been demonstrated in dentinal tubules (Sen *et al.* 1995), failing root canal treatment (Molander *et al.* 1998), and in periapical tissues (Tronstad *et al.* 1987). Prevalence of fungi in infected root canal system ranges from 0.5% to 26% in untreated root canals (Baumgartner *et al.* 2000) and 3.7% to 33% in cases of failed root canal treatment (Molander *et al.* 1998, Waltimo *et al.* 1997).

Most commonly isolated fungus is *Candida albicans* (Waltimo *et al.* 1997). At present, the role of fungi in the pathogenesis of endodontic diseases is still not clear. Similarly, the presence of virus in the infected root canal system has only shown in case reports (Glick *et al.* 1989) and their role in the development of apical lesion is not known.

Taken together, studies strongly support the primary role of bacteria in the development of apical periodontitis and the infected root canal flora is polymicrobial and predominately anaerobic.

1b. Endodontic Therapy

The goal of endodontic treatment is to prevent and eliminate apical periodontitis. This objective is accomplished through disinfection of the root canal space and adequate root canal filling to prevent recontamination. The endodontic treatment protocol should be based on sound biologic rationale for more effective clinical management of the disease.

Long-term endodontic success for the teeth with apical periodontitis ranges from 75% to 90% (Stringberg *et al.* 1956, Kerekes and Tronstand 1979, Byström *et al.* 1987, Sjogren *et al.* 1990, Trope *et al.* 1999, Weiger *et al.* 2000, Peters & Wesslink 2002) with functional success rate of 95% (Byström *et al.* 1987, Weiger *et al.* 2000, Peters & Wesslink 2002). Endodontic success (healed) is defined as the absence of clinical symptoms and radiographic resolution of periapical lesion (Stringberg *et al.* 1956).

In a retrospective study with 6-month to 5-year follow-up, 478 teeth endodontically treated by undergraduate students were evaluated (Kerekes and Tronstand 1979). Overall success rate (both vital and non-vital cases) was 83% at first year, then, increased to 91% at 3-5 year follow-up. In cases of apical periodontitis, the success rate was 85%. Other

retrospective studies also demonstrated similar success rate for the teeth with apical periodontitis. Stringberg *et al.* (1956) showed 80% healed at 6-month to 10-year follow-up. Sjögren *et al.* (1990) had 86% success rate with 8-10 year follow-up period. Toronto study (Farzaneh *et al.* 2004) showed 79% success rate at 4-6 year follow-up.

Prospective studies also showed comparable success rate. Sjögren *et al.* (1997) examined the 5-year outcome study on 53 single-rooted anterior teeth with radiographic periapical lesion. Overall success rate was 83%. In another prospective study, 79 endodontically treated teeth with apical periodontitis were followed up for 2 to 5 years (Byström *et al.* 1987). Eighty five percent (85%) of the cases were healed completely and 9% of the cases were healing, with functional success rate of 94%. In agreement with this finding, other studies also showed about 95% functional success rate for endodontically treated teeth (Weiger *et al.* 2000, Peters & Wesslnik 2002).

In contrast to the university-based study, cross-sectional studies showed much lower success rate. Buckely and Spangberg (1995) assessed the prevalence of radiographically detectable periapical disease and its relationship to the quality of endodontic care in a North American population. The authors examined 208 randomly chosen full-mouth x-ray (FMX) series with 5,272 teeth taken at the initial screening. They found 31.3% of root canal filled teeth had periapical disease and only in about 40% of cases was technically satisfactory root canal treatment achieved. Similarly, Ray and Trope (1995) evaluated 1,010 endodontically treated teeth from FMX series with minimal of 1 year follow-up. Overall, they found 61% of the teeth had no radiographic sign of apical periodontitis. Lower success rate observed in cross-sectional studies is likely due to the poor quality of endodontic care performed in private practice setting.

Long term success of endodontic therapy depends on many factors including tooth vitality, level of root-canal disinfection, length and quality of root canal filling, and coronal restoration (Stoll *et al.* 2005, Schaeffer *et al.* 2005, Caplan & Weintraub 1997, Sorensen *et al.* 1984, 1985, Vire *et al.* 1991, Aquilino & Caplan 2002, Ray & Trope 1995).

Outcome studies of root canal treatment consistently showed higher long-term success rate for the teeth without apical periodontitis than the teeth with apical periodontitis (Sjögren *et al.* 1990, Kerekes & Tronstad 1979, Stringberg *et al.* 1956, Stoll *et al.* 2005). Teeth with vital pulp have long-term success rates of 95-97%, while teeth with apical periodontitis range from 75% to 90% (Stringberg *et al.* 1956, Kerekes and Tronstad 1979, Byström *et al.* 1987, Sjögren *et al.* 1990, Trope *et al.* 1999, Weiger *et al.* 2000, Peters & Wesslink 2002). The observed difference in the outcome between these two groups is due to the ability of operators to disinfect root canal system. In case of vital pulp, root canal space is not infected with microorganism but filled with inflamed pulp tissue (Seltzer and Bender 1963, Mumford *et al.* 1967, Dummer *et al.* 1980). Hence, it is more likely to achieve a root canal space free of bacteria assuming the treatment is performed under strict aseptic protocol. On the other hand, teeth with apical periodontitis are usually associated with necrotic pulp infected with obligate and facultative bacteria (Sundqvist 1976, Möller *et al.* 1981, Fabricius *et al.* 1981). In such case, complete elimination of bacteria from the root canal space is limited due to the complexity of root canal anatomy and resistant bacteria.

Sjögren *et al.* (1997) investigated the role of infection on the prognosis of endodontic therapy by following-up teeth that had their canals cleaned and obturated during a single appointment. In this study, the root canals of 55 single rooted teeth with apical periodontitis were thoroughly instrumented and irrigated with NaOCl solution. Periapical healing was

followed up for 5 years. Complete healing occurred in 94% of cases that yielded negative culture. Where the samples were positive prior to root filling, the success rate of treatment was just 68%. Byström *et al.* (1987) also demonstrated 95% healing when the root canal filled after the negative culture. Other studies also demonstrated similar results (Zeldow & Ingle 1963, Engström *et al.* 1964). Hence, the presence of bacteria prior to root canal filling is a negative predictor for the long term success of root canal treatment.

Root canal disinfection can be accomplished by mechanical and chemical means. Mechanical microbial control phase involves root canal instrumentation using hand and/or rotary instruments. In contrast, chemical microbial control phase involves antimicrobial agents during and after instrumentation.

Mechanical instrumentation can reduce intra-canal bacteria significantly but can not predictably eradicate them. Byström and Sundqvist (1981) evaluated the efficacy of the mechanical instrumentation in bacterial reduction during root canal treatment. Fifteen single-rooted teeth with apical periodontitis were treated in five visits with no inter-appointment dressing. Root canals were instrumented with hand files and with physiological saline as irrigant. Mechanical instrumentation with saline reduced the number of bacteria significantly, by 100-1000 fold. However, despite being treated 5 times, bacteria can not be predictably eliminated from the root canal system. They also showed that 0.5% NaOCl was more effective than physiological saline when used as irrigant. Other studies also support this finding (Siqueira *et al.* 2000, Byström & Sundqvist 1985, Dalton *et al.* 1998, Shuping *et al.* 2000)

On average, 40% - 60% of root canals have no cultivable bacteria after mechanical root canal instrumentation with NaOCl solution (Dalton *et al.* 1999, Shuping *et al.* 2001,

Card *et al.* 2002, McGurkin-Smith *et al.* 2005, Kvist *et al.* 2004). Shuping *et al.* (2000) evaluated the extent of bacterial reduction with NiTi rotary instrumentation and 1.25% NaOCl irrigation in 42 patients with apical periodontitis. Bacterial samples were taken before and after the instrumentation, and they were cultured anaerobically for 7 days. The authors found 62% of canals were rendered bacteria-free after instrumentation with 1.5% NaOCl. McGurkin-Smith *et al.* (2005) obtained 40% of canals sampled bacteria free after instrumentation and a strict irrigation protocol using 5.25% NaOCl and EDTA.

Another method to improve root canal disinfection is to enlarge the apical preparation (Dalton *et al.* 1999, Siqueira *et al.* 1999, Shuping *et al.* 2000, Card *et al.* 2002, Baugh & Wallace 2005). Dalton *et al.* (1999) demonstrated that regardless of technique (hand file or rotary file), uniform reduction of bacteria occurred with progressive filing. Siqueira *et al.* (1999) showed the same result from *in vitro* study. In a clinical study, Card (2002) was able to demonstrate 100% of canine and premolar, and 89% of molars rendered bacteria free after preparing apical size to #60 and #80 using LightSpeed instruments. In addition to mechanical reduction of bacteria, apical enlargement also allows better access for the irrigant to reach the apical region and disinfect the bacterial contents (Shuping *et al.* 2000, Salzgeber *et al.* 1977, Ram *et al.* 1977, Chow *et al.* 1983, Siqueira *et al.* 1999). Studies have shown that canals need to be enlarged to at least #35 file for adequate irrigation to reach apical third. Ram *et al.* (1977) concluded that canals need to be enlarged to a #40 file size so that maximum irrigation is in contact with the apical debris. Chow *et al.* (1983) also showed the canal system had to be instrumented to at least #40 file for proper irrigation. Shuping *et al.* (2000) and Siqueira *et al.* (1999) later confirmed the findings that NaOCl requires a certain size canal to become beneficial in bacterial reduction.

Nonetheless, eradication of endodontic infection by instrumentation and NaOCl solution alone is limited (Byström & Sundqvist 1981, 1985, Sjögren *et al.* 1991, Dalton *et al.* 1999, Shuping *et al.* 2000, Card *et al.* 2002). To obtain a more predictable microbial control, placement of intracanal medicaments such as calcium hydroxide ($\text{Ca}(\text{OH})_2$) is recommended (Ørstavik *et al.* 1991, Shuping *et al.* 2000, Sjögren *et al.* 1991). Intracanal medicaments are used to: (a) eliminate bacteria in the root canal; (b) prevent bacterial proliferation between appointments; and (c) act as a physicochemical barrier, preventing root-canal reinfection and nutrient supply to the remaining bacteria (Byström & Sundqvist 1985). Calcium hydroxide is one of most widely used intracanal medications in endodontics today and remains the best medicament available to reduce residual microbial flora (Law *et al.* 2004). It is a strong alkaline substance, which has a pH of approximately 12.5. In an aqueous solution, calcium hydroxide dissociates into calcium and hydroxyl ions that will lead to a lowered oxygen tension and an increase in the pH in the inflamed periapical tissues (Siqueira *et al.* 1999). With a high pH, calcium hydroxide has an excellent broad antimicrobial effect.

To support this, studies consistently demonstrated that $\text{Ca}(\text{OH})_2$ can help to further eliminate surviving bacteria in the root canals. Sjögren *et al.* (14) showed that bacteria were not found in teeth with apical periodontitis dressed with $\text{Ca}(\text{OH})_2$ for 1 wk. Ørstavik *et al.* (1991) demonstrated that extensive apical reaming and 1 week dressing with $\text{Ca}(\text{OH})_2$ significantly reduced bacterial growth from root canal and apical dentin samples. Shuping *et al.* (2000) showed that placement of $\text{Ca}(\text{OH})_2$ for at least 1 week rendered 92.5% of canals bacteria free in teeth with apical periodontitis. Law *et al.* (2004) reviewed the literature evaluating the antibacterial effectiveness of $\text{Ca}(\text{OH})_2$ used in the management of apical

periodontitis and found 27% of canals showed detectable bacteria growth after medication. However, $\text{Ca}(\text{OH})_2$ has been shown to be ineffective when used as a short-term dressing (< 1 d) (Sjögren *et al.* 1991, Safavi *et al.* 1990) and requires at least 1 week to properly exert its antibacterial action. Thus, a minimum of two-visit root canal treatment is strongly recommended for adequate intracanal disinfection in cases of apical periodontitis (Trope *et al.* 1991).

1c. Sodium Hypochlorite (NaOCl)

Sodium hypochlorite was first recommended as an antiseptic solution by Henry Dakin (1915) to irrigate open wounds during World War I. Later, Coolidge (1919) introduced NaOCl to endodontics and it has remained the most used irrigant.

NaOCl has several characteristics that make this solution a desirable endodontic irrigant, including its antimicrobial (Byström & Sundqvist, 1983, 1985, Siqueira *et al.* 2000, Zehnder *et al.* 2002), tissue dissolving (Thé *et al.* 1980, Moorer & Wesselink 1982, Zehnder *et al.* 2002) and lubricating properties.

NaOCl has a broad-spectrum antimicrobial activity (Byström & Sundqvist 1983). It can kill vegetative bacteria, spore-forming bacteria, fungi, protozoa, and viruses (Rutala & Weber 1997). Effectiveness of NaOCl as intracanal antimicrobial agent has been well documented.

There have been conflicting results concerning the antimicrobial efficacy of different NaOCl concentrations. In general, *in vitro* studies (Siqueira *et al.* 1998, Yesilsoy *et al.* 1995) showed that 5.25% NaOCl is more effective than lower concentration solution in eliminating bacteria. In contrast, clinical studies found no difference in the antimicrobial effect between 0.5% and 5% NaOCl (Byström & Sundqvist 1985, Cvek *et al.* 1976). This disparity between

in vitro and clinical studies is likely attributable to the differences in methodology. *In vitro* studies for testing antimicrobial properties of a solution are often carried out in test tubes or agar plates, which do not take into consideration of root canal complexity. To eliminate bacterial cells within the root canal system, irrigants must be able to reach them. Many areas in the root canal system have the potential to harbor microorganisms, thus making them inaccessible to the effects of chemomechanical preparation. In case of using weaker NaOCl solution, many authors recommended frequent replenishing of the irrigants in order to compensate for the quick neutralization of NaOCl (Byström & Sundqvist 1985, Siqueira *et al.* 2000).

The tissue dissolving ability of NaOCl has been well investigated. Grossman and Meiman (1941) showed that chlorinated soda (solution containing NaOCl and NaCl) was an effective solvent of pulp tissue in an *in vitro* study. It was found to dissolve the pulps of freshly extracted teeth in less than 24 hours. Since then, numerous studies have been published on the tissue dissolution property of NaOCl when used as endodontic irrigant (Moorer & Wesselink 1982, Thé *et al.* 1979, Zender *et al.* 2002). There are several factors thought to be important for the efficacy of NaOCl irrigant in dissolving pulp tissue. In an *in vitro* study, Moorer & Wesselink (1982) investigated the effect of fluid flow, pH and available surface area of tissue on the tissue dissolving capability of NaOCl. The authors concluded that the tissue-dissolving power of NaOCl solution appeared to be strongly depended on (1) the amount of organic matter in the hypochlorite/tissue system (2) the frequency and intensity of mechanical agitation (fluid flow) and (3) the available surface area of free or enclosed tissue. For hypochlorite solution to be effective, it should act quickly and be in an excess in relation to the amount of organic material that was to be digested. In

addition, the authors suggested that it was the amount of NaOCl that was important for the tissue dissolution rather than the concentration. Hence, they recommend the continuous and intensive mechanical debridement of the canal system and repeated refreshment of the NaOCl. These authors also showed the importance of mechanical agitation on tissue dissolving ability of NaOCl, particularly ultrasonic agitation shown to be very effective.

Although higher concentrations of NaOCl show more effective tissue dissolving properties on a fixed surface area (Zehnder *et al.* 2002, Moorer & Wesselink 1982), it is not true when there are only small amounts of tissue (Moorer & Wesselink 1982). Baumgartner and Cuenin (1992) showed that NaOCl in concentration of 1%, 2.5%, and 5.25% completely removed pulpal remnants and predentin from uninstrumented dentinal walls. Thus it appears that low concentration of NaOCl retains adequate tissue dissolving and antibacterial properties.

Cytotoxicity of NaOCl is a major disadvantage for its clinical use. Pashley *et al.* (1985) had tested the cytotoxicity of various dilutions of sodium hypochlorite on 3 independent biological models (hemolysis of RBC, rabbit eye experiments, and intradermal injections). All dilution caused hemolysis, moderate to severe irritation to rabbit eyes, and skin ulceration. It was found that higher concentration NaOCl causes more cytotoxic effects on the vital tissue. The authors concluded that 5% NaOCl must be used judiciously and with great caution to prevent it from reaching the periapex where it could elicit severe inflammatory reactions. Spangberg *et al.* (1973) suggested that 5.25% NaOCl was considerably stronger than necessary to eliminate bacterial strains commonly found in infected root canals. At this concentration, the author believed NaOCl was considered too cytotoxic to be routinely used in endodontic therapy.

Taken together, low concentration of NaOCl is sufficient to dissolve pulp tissue and eliminate bacteria in the root canal system. Clinical use of full-strength NaOCl should be avoided due to its strong cytotoxicity.

1d. Smear Layer Removing Agents

Current methods of root canal instrumentation produce a smear layer on the surface of canal walls (McComb & Smith 1975, Goldman *et al.* 1988, Yamada *et al.* 1983, Torabinejad *et al.* 2002). Under Scanning Electronic Microscopy (SEM), this layer has amorphous, irregular, and granular appearance that can penetrate into and occlude the dentinal tubules (McComb & Smith 1975). It contains both inorganic and organic substances that include dentine, remnant of pulp tissue, odontoblastic processes, and microorganisms (Torabinejad *et al.* 2002).

In infected root canals, bacteria, predominantly Gram-negative anaerobes, can be identified from the root canal system and in the dentinal tubules (Ando & Hoshino 1990, Sen *et al.* 1995, Love & Jenkinson 2002). Viable bacteria were isolated from the infected dentinal tubules of root canal system in extracted human teeth (Peters *et al.* 2001). Histological and SEM studies showed bacteria can penetrate into the dentinal tubules at various distances from the surface of root canal wall (Ando & Hoshino 1990, Sen *et al.* 1995, Peters *et al.* 2001). In some case, bacteria can potentially penetrate to the cementum layer (Peters *et al.* 2001). Under *in vitro* conditions, several investigators have successfully infected the dentinal tubules with microorganisms (Haapasalo & Ørstavik 1987, Siqueira *et al.* 1996, Perez *et al.* 1993). Hence the available evidence strongly suggests that viable bacteria can invade the dentinal tubule during root canal infection process.

The presence of smear layer after root canal instrumentation raises some concerns. Several investigators have found that the smear layer itself may be infected and may protect bacteria already in the dentinal tubules (McComb & Smith 1975, Haapasalo & Ørstavik 1987). It has been shown that the smear layer is not a complete barrier to bacteria and it delays but does not abolish the action of endodontic disinfectants (Ørstavik & Haapasalo 1990). Clinically, whether bacteria in the dentinal tubule can be predictably eliminated by the disinfectants, in the presence of smear layer, is questionable. The remaining viable bacteria in the dentinal tubule may be responsible for the persistent endodontic infection after root canal therapy (Stuart *et al.* 2006). Hence, it is prudent to remove the initially created smear layer in infected root canals and to allow penetration of endodontic disinfectants into the dentinal tubule more effectively (Torabinejad *et al.* 2002).

The smear layer of root canal walls also act as a physical barrier interfering with adhesion and penetration of sealers into dentinal tubules (Abramovich & Goldberg 1976, Tidmarsh 1978, White *et al.* 1984). Studies have shown that root canal sealers can penetrate into dentinal tubules in the absence of smear layer; whereas, no penetration was observed with the smear layer intact (Gutiérrez *et al.* 1990, Pallarés *et al.* 1995, Kouvas *et al.* 1998). This characteristic is particularly important for some root canal filling materials such as Resilon (Shipper *et al.* 2004) and many other resin-based sealers. Removal of smear layer prior to root canal filling has also shown to reduce microleakage (Pashley & Depew 1986, Taylor *et al.* 1997, Timpawat *et al.* 2001).

EDTA (Ethylenediaminetetracetic acid) is a chelating agent, commonly used for removal of smear layer from the instrumented root canal walls (McComb & Smith 1975, Yamada *et al.* 1983, Baumgartner & Mader 1987). Since smear layer is composed of both

organic and inorganic materials, the combination of EDTA and sodium hypochlorite (NaOCl) is often recommended (Goldman *et al.* 1988, Yamada *et al.* 1983, Baumgartner & Mader 1987). In addition to EDTA, citric acid can also be used (Baumgartner *et al.* 1984). Recently, new solutions have been proposed with enhanced antimicrobial properties in addition to their smear layer removal ability such as BiopureTM MTAD (Torabinejad 2003a, Torabinejad 2003b) and SmearClearTM. Although their abilities to remove smear layer are well demonstrated, antimicrobial efficiency of BiopureTM MTAD has been questioned (Kho and Baumgartner 2006, Baumgartner *et al.* 2007)

1e. Calcium hydroxide (Ca(OH)₂)

Calcium hydroxide was introduced in endodontic therapy by Hermann in 1920 and often recommended for disinfection of the root canal space in teeth with apical periodontitis.

The effectiveness of interappointment Ca(OH)₂ has been well investigated. Byström *et al.* (1985) reported that Ca(OH)₂ was an effective intracanal medicament rendering 34 out of 35 canals bacteria free after 4 weeks. Sjögren *et al.* (1991) who demonstrated that a 7-day dressing with Ca(OH)₂ eliminated all bacteria in the root canal but it is ineffective when used for short-term. Shuping *et al.* (2000) also demonstrated the effectiveness of Ca(OH)₂ as root canal disinfectant when used for > 1 week.

Even though Ca(OH)₂ is a good antimicrobial agent, it is ineffective against some of microorganisms such as *Enterococcus faecalis* (Sjögren *et al.* 1991, Haapasalo and Ørstavik 1987, Heling *et al.* 1992), which is found in the case of persistent root canal infection. Byström *et al.* (1985) showed the most resistant strain to Ca(OH)₂ belonged to the genus *Enterococcus*, which can survive at pH 11.5, but not at pH 12.5. In addition, some studies

have questioned the effectiveness of Ca(OH)_2 to disinfect the canal and reported a residual flora in the canal after one or more weeks with Ca(OH)_2 (Reit *et al.* 1999, Peters *et al.* 2002, Kvist *et al.* 2004, Waltimo *et al.* 2005). Hence, it is necessary to explore new antimicrobial agents that are effective in eliminating intracanal microorganisms including facultative microorganisms such as *E. faecalis*.

1f. Chlorhexidine (CHX)

Despite the fact that NaOCl eradicates bacteria effectively, it is caustic if accidentally expressed into the periapical area or adjacent structures (Hülsmann and Hahn 2000). In addition, it has strong bleaching effect and foul odor. Chlorhexidine (CHX) gluconate has been suggested as an alternative irrigating solution that could replace NaOCl.

CHX is one of the most widely used biocides in antiseptic products in general. It is a cationic bisguanide that seems to act by adsorbing onto the cell wall of microorganism and cause leakage of intracellular components. At low concentration, it has bacteriostatic effect. While at high concentration, it is bactericidal due to precipitation and/or coagulation of intracellular constituents (McDonnell and Russell 1999). Its optimal antimicrobial activity is at pH 5.5-7.0 (Russell and Day 1993).

CHX gluconate has been in use for a long time in dentistry. It has a broad spectrum antimicrobial activity, targeting both Gram positive and Gram negative bacteria (Delany *et al.* 1982, Ringel *et al.* 1982, Jeansonne & White 1994). In general, *in vitro* studies suggested that CHX and NaOCl have comparable antibacterial effect when used in similar concentration (Jeansonne & White 1994, Estrela *et al.* 2003, Vianna *et al.* 2004). Jeansonne and White (1994) compared the antimicrobial activity of 2.0% CHX gluconate with that of

5.25% NaOCl in an *in vitro* root canal system. Microbiological samples were taken before and after instrumentation with each endodontic irrigant. The number of positive cultures and colony-forming units after instrumentation were lower in CHX-treated than NaOCl-treated teeth, but the differences were not statistically significant. Estrela *et al.* (2003) tested the minimum inhibitory concentration and antimicrobial effectiveness by the direct exposure of endodontic irrigants for microbes commonly isolated from the infected root canal system and found that 2% CHX and 5.25% NaOCl had similar antimicrobial activity against those organisms. Vianna *et al.* (2004) showed the time required for 1.0% and 2.0% CHX liquid to eliminate *Staphylococcus aureus*, *Candida albicans*, *E. faecalis*, *Prevotella endodontalis*, *Porphyromonas gingivalis*, and *Prevotella intermedia*, was the same as required for 5.25% NaOCl.

In addition to its antimicrobial property, CHX has substantivity in root canal dentin (Basrani *et al.* 2002, Rosenthal *et al.* 2004, Dametto *et al.* 2005). Rosenthal *et al.* (2004) investigated the substantivity of CHX in bovine roots. Roots with standardized length were instrumented with 1% sodium hypochlorite and EDTA followed by placing in 2% CHX solution for 10 minutes prior to obturation. After a certain period, all specimens were halved and canal wall dentin was ground out with Peeso reamers followed by mixing the dentin debris with *E. faecalis* in a test tube. It was shown that CHX can be retained in root canal dentin in antimicrobial effective amounts for up to 12 weeks. Other studies also demonstrated the substantivity of CHX in root canal dentin (Basrani *et al.* 2002, Dametto *et al.* 2005).

The relatively low toxicity of CHX is an additional advantage. Filho *et al.* (2002) evaluated the inflammatory response to irrigation solutions (0.5% NaOCl and 2% CHX)

injected into the peritoneal cavity of mice. They demonstrated 0.5% NaOCl solution induced inflammatory response while 2% CHX solution did not induce a significant inflammatory response. In a recent study, Dammaschke et al. (2005) assessed the effects of 2% CHX gel and calcium hydroxide on apical periodontitis in Wistar rats. The investigators showed that CHX gel used as intra-canal medicament led to good periapical regeneration, suggesting that this may be an alternative to calcium hydroxide root canal dressing.

Despite the advantages of CHX, its activity is pH dependent and is greatly reduced in the presence of organic matter (Russell and Day 1993). Unlike sodium hypochlorite, it lacks tissue dissolving properties (Naenni *et al.* 2004).

2% CHX in a gel formulation (Endogel, Itapetininga, SP, Brazil) was proposed as an alternative to 5.25% NaOCl solution for root canal disinfection (Ferraz *et al.* 2001). This lubricant is composed of a gel base (1% natrosol) which is a nonionic, highly efficient, inert, water-soluble agent, and 2% CHX gluconate at pH 7.0. Ferraz *et al.* (2001) assessed the CHX gel as an endodontic irrigant *in vitro*. The results indicated that the CHX gel produced a cleaner root canal surface and had an antimicrobial activity comparable with that obtained with the other solutions (NaOCl and CHX liquid) tested. It was concluded that chlorhexidine gluconate in gel form has potential for use as an endodontic lubricant. Dametto *et al.* (2005) assessed *in vitro* the antimicrobial activity of 2% CHX gel against teeth infected with *E. faecalis*, comparing it to other endodontic irrigants (2% CHX liquid and 5.25% NaOCl). The results showed that the antimicrobial abilities of 2% CHX gluconate, gel and liquid form, were more effective than 5.25% NaOCl in suppressing CFU of *E faecalis* for 7 days after the biomechanical preparation. In another study, Gomes *et al.* (2003) showed that 2% CHX gel alone was more effective against *E. faecalis* than calcium hydroxide as an intracanal dressing

in extracted bovine teeth infected with *E. faecalis*. It appeared that CHX in a gel form required a much longer time to kill *E. faecalis* than the corresponding concentration in a liquid (Vianna *et al.* 2004).

There are limited clinical studies available in the literature regarding the effectiveness of 2% CHX in disinfecting root canal system. One study showed that the antibacterial activity of 2% CHX gluconate solution is superior to that of 5.25% NaOCl in infected root canal (Ercan *et al.* 2004). However, there was no statistical difference between two endodontic irrigants, probably due to the small sample numbers. Another study evaluated the addition of a 2% CHX rinse to a conventional treatment protocol in the enhancement of the rate of the successful disinfection of the root canal system (Zamany *et al.* 2004). Cultivable bacteria were recovered in 7 out of 12 cases treated with a conventional protocol (instrumentation with 5.25% NaOCl) while only 1 out of 12 cases treated with a conventional protocol and final rinse with 2% CHX solution had bacterial growth.

CHAPTER 2

PURPOSE

Despite its promising results from *in vitro* studies, clinical studies addressing effectiveness of 2% CHX gel have been limited. The objective of this study was to evaluate the effectiveness of 2% CHX gel to eradicate endodontic infection in patients with apical periodontitis. The additional antibacterial effect of Ca(OH)_2 mixed with 2% CHX gel as a paste for an intracanal dressing was also assessed.

CHAPTER 3

MATERIALS AND METHODS

3a. In Vitro Evaluation of 2% CHX Gel Neutralizer

An effective method of inactivating 2% CHX solution by 3% Tween 80 and 0.3% L- α -lecithin was previously shown in the literature (Zamany and Spångberg 2002). However, whether 3% Tween 80 and 0.3% L- α -lecithin can effectively neutralize 2% CHX gel (Endogel, Itapetininga, SP, Brazil) has not been evaluated. To address this issue, the following *in vitro* study was carried out based on the protocol described by Zamany and Spångberg (2002).

Bacterial Cell Suspensions

E. faecalis was cultivated on blood agar medium for 24 hours. Colonies were harvested from the surface of the agar plate and suspended in a 0.43% solution of sodium chloride, centrifuged at about 400g for 10 minutes, washed twice, and resuspended. They were centrifuged and finally resuspended in 0.43% solution of sodium chloride. A stock cell suspension with a density of 2×10^4 viable cells per mL was prepared. The cell density was determined by measuring light absorbance with a spectrophotometer (Ultrospec LKB Biochrom, Rochester, NY) at a wavelength of 580 nm according to MacFarland's scale (Remel, Lenexa, Kan). Utility cell suspensions were prepared by diluting the stock cell

suspension in 0.43% solution of sodium chloride. Each cell suspension was used within 30 minutes of preparation.

Test Procedure

Three percent (3%) Tween 80 plus 0.3% L- α -phosphatidylcholine (L- α -lecithin) was prepared by dissolving 0.75g of L- α -lecithin (Sigma Chemical) in 3 mL of Tween 80, and adjusting the final volume by adding 97 mL of 0.43% sterile saline solution.

1 mL of 2% CHX gel (Endogel, Itapetinga, SP, Brazil) was tested against 9 mL of 3% Tween 80 plus 0.3% L- α -lecithin at room temperature (25°C) for 5 min in a sterile test tube containing glass beads (diameter, 3 mm). 0.1 mL of bacterial cell suspension, containing 2×10^4 viable cells was added to this mixture. At 10 and 60 minutes, 3 glass beads and 0.1 mL aliquots were withdrawn and spread over the surface of two blood agar plates by means of the glass beads, which were incubated at 37°C for 72 hours. The numbers of colony-forming units (CFU) on the blood agar plates were recorded. The experiment was repeated and the mean CFUs from two experiments were calculated and recorded. To observe the storage life of the neutralizing agent, the same experiment was carried out weekly for a period of 3 months.

To test the effectiveness of 2% CHX gel, 0.1 mL of bacterial cell suspension was tested against the mixture of 9mL of 0.43% sterile saline and 1 mL of 2% CHX gel, which was equilibrated for 5 min.

To confirm that 3% Tween 80 plus 0.3% L- α -lecithin did not have antimicrobial effect, 0.1 mL of bacterial cell suspension was tested against the mixture of 9mL of 0.43% sterile saline and 1 mL of the neutralizing agent which was equilibrated for 5 min.

For the positive control, 0.1 ml of bacterial cell suspension was added to the mixture of 10 mL of 0.43% sterile saline.

3b. Subject Recruitment and Qualification

Approval for the project was obtained from the University of North Carolina School of Dentistry Committee on Investigation Involving Human Subjects. Patients presenting to the University of North Carolina School of Dentistry graduate endodontic clinic for evaluation and treatment of infected pulps with apical periodontitis as verified radiographically were considered for this study. The primary investigator conducted all clinical and sampling procedures. Patient selection and treatment began in January 2006 and ended in December 2006. The nature of study, complications and associated risks were fully explained to the patients or patients' guardians and consents were obtained prior to the treatment (**Appendix 1**). Each patient was assigned a reference number that only the primary investigator knew. References to individual treatment were by reference number to maintain confidentiality.

All teeth types were included in the study. The mesio-buccal roots of the mandibular first and second molars, the distal roots of maxillary first and second molars, the buccal roots of maxillary first premolars, and single rooted teeth were sampled and included in the study. Inclusion criteria were:

- Radiographic evidence of a periapical radiolucent lesion associated with tooth.
- Necrotic pulp as indicated by thermal or electric pulp testing.
- No history of previous endodontic treatment of the tooth
- Enough crown structure for adequate isolation

Exclusion criteria were:

- Teeth with unfavorable conditions for rubber-dam application.
- Vital pulp tissue observed during the treatment.
- Immature teeth with open apices

3c. Treatment Group Assignment

Qualified subjects were accepted into the study in a non-random consecutive sample and treated with ProFile 0.04 taper nickel-titanium files (Dentsply/Tulsa Dental, Tulsa, Oklahoma) and 2% CHX gel as disinfectant. Upon the completion of root canal instrumentation at the end of first appointment, all teeth were dressed with Ca(OH)_2 mixed with 2% CHX gel for at least 2 weeks. In this study, forty-three patients diagnosed with apical periodontitis were included as the test subjects and four patients with irreversible pulpitis as the negative control subjects.

3d. Bacteria Sampling

Each tooth was isolated with rubber dam and disinfected with 30% hydrogen peroxide until no further bubbling of the peroxide occurred. If difficulty occurred in attaining a bubble-free status, Oraseal Putty (Ultradent Products Inc., South Jordan, UT) was placed around the neck of the tooth and the process repeated. All surfaces were then coated with tincture of iodine and allowed to dry. Gross caries removal and initial access form were accomplished with sterile high speed and low speed burs. The rubber dam and surrounding tooth structure were disinfected with iodine tincture before completing the access with another sterile bur. After access was achieved, cases were randomly selected for sterility

testing of the operating field. The tooth surface was swabbed with a 5% sodium thiosulfate solution to inactivate the iodine tincture so that residual iodine would not influence bacteriologic sampling (Pocock 1983). To assess the efficacy of the disinfection procedure, a sterile cotton pellet was moistened in 5% sodium thiosulfate solution and used to swab the access cavity. The swab was then transferred to a vial containing 1 ml of liquid dental transport medium (LDTM) (Anaerobe Systems, Morgan Hill, CA) and sampled for bacterial growth. Sterile saline was used to flush debris within the chamber. A description of the 3 samples (S1, S2, S3) taken in the study is found in **Table 1**.

Initial Sample (S1)

Bacteria samples were collected from the mesiobuccal canals of mandibular molars, distobuccal canals of maxillary molars, buccal canals of upper premolars, and single-rooted teeth. In multi-rooted teeth, sterile orifice openers (Dentsply/Tulsa Dental) were used to open the orifices of canals not used in this study, and those canal orifices were sealed with Cavit (ESPE, Norristown, PA) until all samples collected. A new sterile orifice opener (Dentsply/Tulsa Dental) was used to initiate the access into the canals of interest. Sterile saline was again used to flush any debris from the chamber. The chamber was dried with sterile cotton pellets and/or paper points before placement of Liquid Dental Transport Media (LDTM) (Anaerobic Systems, Morgan Hill, CA) into the canals of interest with a sterile tuberculin syringe. The canals were instrumented to size ISO #15 with sterile stainless steel K-file (Kerr, Romulus, MI) to within 1 mm of the estimated working length. The LDTM remaining in the canal was soaked up and transferred to the LDTM vial with sterile xx-fine paper points (Mynol, Block Drug Corp., Jersey City, NJ) placed as close to working length as possible. This constituted the initial sample (S1). All samples were reached the laboratory within 24 h. The description of initial sampling is shown in **Figure 1**.

Instrumentation

Working length was established to the root terminus using an apex locator (Root ZX®, J. Morita, Irvine, CA) and confirmed radiographically, 0.5-1.0mm short of radiographic apex. The canals and pulp chambers were filled with 2% CHX gel using 30 gauge Maxi-Probe irrigation needles (Dentsply Int. Inc., York, PA) before instrumentation. Sterile ProFile 0.04 taper nickel-titanium files (Dentsply/Tulsa Dental, Tulsa, Oklahoma) were used to instrument the canals by a crown-down technique to standardized apical sizes. Rotary instrumentation was used with an Aseptico ITR Electric Torque Control Motor (Dentsply/Tulsa Dental, Tulsa, Oklahoma) rotating at 300 rpm. The mesiobuccal canals of mandibular molars, distobuccal canals of maxillary molars, and buccal canals of upper premolars were instrumented to apical size #40.04. All single-rooted teeth were instrumented to apical size #60.04. The apical preparation size based on the tooth type is shown in **Table 2**. The canals were irrigated with saline using a 30 gauge Maxi-Probe irrigation needles (Dentsply International, PA) followed by replenishing with 2% CHX gel between files. After the instrumentation, the canal spaces were filled with 2% CHX gel for 2 min followed by final rinse with 10mL of physiological saline.

Post-instrumentation Sample (S2)

The canals were flushed with 2ml of 3% Tween 80 plus 0.3% L- α -lecithin (Sigma Corp, St. Louis, MO) (Zamany and Spångberg 2002) to neutralize 2% CHX gel. The canals were then flushed with 2ml sterile saline and dried with sterile paper points. Using a new set of sterile instruments, the canals were filled with LDTM and the final file sizes were placed to the working length. Files were pumped five times with minimal reaming motion. The entire canal content were absorbed with sterile paper points and transferred to the LDTM sample vial. This constituted the post-instrumentation sample (S2). All samples reached the

laboratory within 24 h. Once samples were collected from the canals of interest, Cavit (ESPE, Norristown, PA) was removed from the remaining canals followed by instrumentation with 2% CHX gel. The description of post-instrumentation sampling is shown in **Figure 2**.

Intracanal medicament

The canals were again filled with 2% CHX gel for 2 min, then saline and dried with paper points. A mixture of Ca(OH)_2 and 2% CHX gel was placed into all canals with a Lentulo spiral filler (Caulk, Milford, Delaware) and the access cavity was sealed with IRM (Dentsply Int. Inc., York, PA). The intracanal medication of the Ca(OH)_2 mixed with 2% CHX gel was placed for a minimum of two weeks.

Post-Dressing (S3)

At the second appointment, under rubber dam isolation the tooth was accessed with the strict aseptic protocol described above. Intracanal medicament was passively removed with a K-file and sterile saline irrigation. The remaining canals were again sealed with Cavit (ESPE, Norristown, PA) till the sample collected from the canal of interest. Neutralization of the Ca(OH)_2 /2% CHX gel dressing was accomplished with 2 ml 0.5% citric acid followed by 2ml 3% Tween 80/0.3% L- α -lecithin introduced into each canal with a sterile tuberculin syringe with 30 gauge Maxi-Probe irrigation needle (Dentsply Int. Inc., York, PA). The canals were irrigated again with sterile saline and dried. As described above, LDTM was introduced and collected, constituting the final sample (S3). The description of post-dressing sampling is shown in Figure 3.

The laboratory procedures were performed at the University of North Carolina Dental Microbiology Laboratory (a CLIA certified laboratory). The vials with the paper point samples were agitated with a vortex with a setting of four before aliquot disbursement.

Sample dilutions of 10 and 100-fold were prepared under anaerobic conditions using sterile glassware. Petri dishes with anaerobic sheep blood agar supplemented with heme and vitamin K were quantitatively inoculated by spiral plating of undiluted sample, as well as each of the two dilutions. A model D spiral plater (Microbiology International) delivered 49µl of sample to each agar plate. The Model D spiral plater delivered a 2.3 log dilution of the sample across each plate. Plates were incubated at 37°C for 7 days in an anaerobic chamber containing 10% hydrogen, 85% nitrogen, and 5% CO₂. The bacteria growth was measured by direct counting of colonies and grid specific calculations. The spiral plater deposited a known volume of the sample to areas of the plate or grid. Once the colonies were counted in each grid, a dilution factor (determined by manufacturer) was used to translate the grid calculations to the original bacterial count in the sample.

3e. Data Analysis

The number of positive culture was also recorded at S1, S2, and S3. Fisher's exact tests were used to detect the differences in the number of positive culture between S1 and S2, S1 and S3, or S2 and S3. The level of significance was set at 0.05 for all analyses.

CHAPTER 4

RESULTS

4a. *In Vitro* Evaluation of 2% CHX Gel Neutralizer

3% Tween 80 plus 0.3% L- α -lecithin was found to be an effective inactivating agent, ensuring full recovery of *E. faecalis* in the presence of 2% CHX gel. The duration of contact time (10 min or 60 min) had no effect on the recovery of the organisms when 3% Tween 80/0.3% L- α -lecithin tested with and without 2% CHX gel. No test organisms were recovered when *E. faecalis* tested against 2% CHX gel (negative control). 3% Tween 80/0.3% L- α -lecithin yielded full recovery of *E. faecalis* (positive control).

The same experiment was repeated weekly for 12 weeks with the same neutralizing agent prepared initially. At the 12th week, full recovery of *E. faecalis* was still obtained when 3% Tween 80 plus 0.3% L- α -lecithin mixed with 2% CHX gel.

4b. *In Vivo* Evaluation of the Clinical Efficacy of 2% CHX Gel

Forty-three test subjects and four negative control subjects were included in the study. Teeth were instrumented to standardized sizes, #40 or #60, depending on the tooth type (**Table 2**). The bacterial counts (CFU/ml) for each sample are listed in **Table 3**. The sample distribution based on the bacterial counts at S1, S2, and S3 are described in **Figure 4-6**. Percentage of root canals with negative culture in the S2 and S3 samples are summarized in

Table 4 and **Figure 7**. All four negative control subjects showed no bacteria growth at S1, S2, and S3.

Bacteria were initially present in 39 of the 43 test teeth. Four test teeth with no initial bacterial growth were excluded from analysis. Of the teeth with positive culture at S1, 18 teeth were prepared to #60 and 21 teeth were prepared to #40.

Of the teeth that were culture positive at S1, 35 out of 39 (89.7%) were negative for culture at S2. For the size #40 and #60 groups, 19/21 (90.5%) and 16/18 (88.9%) had negative culture, respectively. There was no statistical difference in the numbers of negative culture between these two groups at S2 ($p=0.871$).

The average number of days of calcium hydroxide therapy was 19 days with a range of 14 to 29 days. Three samples in S3 were lost during culturing. Of the remaining samples, 33/36 (91.7%) were free of bacteria at S3. For the size #40 and #60 groups, 18/19 (94.7%) and 15/17 (88.2%) had negative culture, respectively. There was no statistical difference in the numbers of negative culture between these two groups at S3 ($p=0.481$). All four samples that were culture positive at S2 were free of bacteria at S3, but three samples that were culture negative at S2 had recoverable CFU at S3.

The Fisher's Exact test showed a significant difference in the numbers of positive culture between S1 and S2 ($p<0.001$) and between S1 and S3 ($p<0.001$). There was no statistical difference in the numbers of negative culture between S2 and S3 ($p=0.692$).

CHAPTER 5

DISCUSSION

Effectiveness of 2% CHX solution or gel as antimicrobial agent was well investigated *in vitro*. It appeared that CHX and NaOCl have similar antimicrobial activity against the common organisms isolated from the root canal system (Estrela *et al.* 2003, Vianna *et al.* 2004). There are several advantages for the clinical use of CHX as root canal disinfectant over NaOCl, including low toxicity (Filho *et al.* 2002), substantivity (Rosenthal *et al.* 2004), and lack of bleaching. Despite its promising results from *in vitro* studies, there are not enough clinical studies published on literature so far. In this study, we evaluated the bacteria reduction activity of 2% CHX gel as root canal disinfectant in patients with apical periodontitis.

An effective method of inactivating 2% CHX solution was shown previously by Zamany and Spångberg (2002). The authors used L- α -lecithin, Tween 80, and sodium thiosulfate in different proportions to prepare 6 potential inactivating solutions. Inactivating agents and 2% CHX solution were tested against *E. faecalis* on blood agar plate. They showed that the combination of 3% Tween-80 and 0.3% L- α -lecithin was the most effective inactivating agent. However whether 3% Tween 80 plus 0.3% L- α -lecithin can effectively neutralize 2% CHX gel (Endogel, Itapetininga, SP, Brazil) had not been evaluated yet. To address this, an *in vitro* experiment was carried out based on the protocol described by

Zamany and Spångberg (2002). In addition, we repeated the same experiment weekly for 12 weeks using the same neutralizing agent prepared. Our data confirmed that 3% Tween 80 plus 0.3% L- α -lecithin is a good neutralizing agent for 2% CHX gel and it is effective at least for 12 weeks.

Although numerous *in vitro* studies evaluated the antimicrobial properties of 2% CHX gel, there is only one clinical study available in the literature. Vianna *et al.* (2006) evaluated the microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. Thirty-two single rooted teeth with necrotic pulp and apical periodontitis (from 32 patients) were selected for this study. One group (n=16) was irrigated with 2.5% NaOCl during the root canal instrumentation while the other group (n=16) was irrigated with 2% CHX gel. Bacterial load was assessed by the use of real-time quantitative-polymerase chain reaction (RTQ-PCR) and the traditional culturing techniques. RTQ-PCR showed that the bacterial reduction was greater in the NaOCl-group than in the CHX-group. According to culture technique 75% of cases were free of bacteria after chemo-mechanical preparation in the NaOCl-group, while 50% of cases were bacteria free in the CHX-group. The authors concluded that 2.5% NaOCl was a more effective root canal irrigant than 2% CHX gel.

In contrast, our results showed that 2% CHX gel was an effective root canal disinfectant and close to 90% of cases were void of recoverable bacteria after the chemo-mechanical preparation. This disparity is probably due to the differences in clinical procedures of the two studies. In our study, all single rooted teeth were prepared to a standardized size of #60/.04 using rotary NiTi instruments while Vianna *et al.* (2006) prepared to the apical size of #35-45 using K-files followed by step back instrumentation.

Previous studies have shown that uniform reduction of bacteria occurred with progressive filing (Dalton *et al.* 1999, Siqueira *et al.* 1999, Shuping *et al.* 2000, Card *et al.* 2000). Higher load of bacterial reduction is expected for a larger apical preparation. Thus, favorable results observed in our study could be due to the combination of larger apical preparation and chemical disinfection property of 2% CHX gel.

In addition, the irrigation methods used for irrigation were different between these studies. Vianna *et al.* (2006) stated that the use of each instrument was followed by irrigation with a syringe containing 1ml of 2% CHX gel and immediately after with 4ml of saline. On the other hand, we filled the root canal with 2% CHX gel and instrumented with a rotary NiTi file followed by rinse with physiologic saline. This procedure was repeated for each file until the final preparation. In addition, the root canals were soaked with 2% CHX gel for 2min after the instrumentation followed by copious rinsing with physiologic saline. The advantages of our approach are to allow 2% CHX gel to have sufficient contact time and physical contact with the root canal walls. Unlike 2% CHX solution, the gel form requires a longer contact time for effective disinfection. *In vitro* studies showed 2% CHX solution and 5.25% NaOCl had similar antimicrobial activities and required about the same amount of time to eliminate microorganisms (Estrela *et al.* 2003, Vianna *et al.* 2004). However, 2% CHX gel requires much longer time to eliminate microorganisms compared to 2% CHX solution and 5.25% NaOCl (Vianna *et al.* 2004).

In addition to the contact time, adequate delivery of 2% CHX gel to the root canal system is an important factor, particularly in curved roots. Unlike the solution, the gel form does not have a good flowing property. It requires additional attention to deliver the gel to the root canal system. This was accomplished in our study by several ways. First, root canal

preparations were performed with rotary NiTi instruments in the canals filled with 2% CHX gel. By this way, the gel was physically delivered throughout the root canal system as the rotary files contacting the walls. Secondly, larger apical preparation would allow adequate lubrication of the apical third (Shuping *et al.* 2000, Ram *et al.* 1977, Chow *et al.* 1983, Siqueira *et al.* 1999). Third, we used 30 gauge Maxi-Probe irrigation needle (Dentsply Int. Inc., York, PA) to deliver 2% CHX gel. Because of its small diameter, the needle was able to be placed close to the working length and allowed the proximity to the anatomical foramen.

On average, 40% - 60% of root canals have no cultivable bacteria after chemo-mechanical root canal preparation with NaOCl solution (Dalton *et al.* 1999, Shuping *et al.* 2001, Card *et al.* 2002, McGurkin-Smith *et al.* 2005, Kvist *et al.* 2004). Shuping *et al.* (2000) evaluated the extent of bacterial reduction with NiTi rotary instrumentation and 1.5% NaOCl irrigation in 42 patients with apical periodontitis. Bacterial samples were taken before and after the instrumentation, and they were cultured anaerobically for 7 days. The authors found 62% of canals were rendered bacteria-free after instrumentation with 1.5% NaOCl. Using similar clinical protocol, McGurkin-Smith *et al.* (2005) obtained 40% of canals sampled bacteria free after instrumentation and a strict irrigation protocol using 5.25% NaOCl and EDTA. It seems that increasing the NaOCl concentration from 1.5% to 5.25% did not increase the percentage of canals sampled bacteria free. On the other hand, our results showed that 89.7 % of canals prepared with 2% CHX gel cultured bacteria free. There was no difference between the canals prepared to #40.04 and #60.04 in the percentage of bacteria reduction (**Figure 7**). It appears that 2% CHX gel is a more effective disinfectant than 1.5-5.25% NaOCl when it is used as in this study (**Figure 8**). Nonetheless, direct comparison among these studies is not possible even though they were done from the same

institution following similar clinical procedures and treatment philosophy. Currently, we are investigating the clinical efficacy of 2% CHX gel vs. 5.25% NaOCl as well.

To obtain a more predictable microbial control, placement of intracanal medicament of Ca(OH)_2 is often recommended (Byström *et al.* 1985, Sjögren *et al.* 1991, Shuping *et al.* 2002). Studies consistently demonstrated that Ca(OH)_2 can help to further eliminate surviving bacteria in the root canals (Sjögren *et al.* 1991, Ørstavik *et al.* 1991, Shuping *et al.* 2002). However, a minimum of two-visit root canal treatment is required when using Ca(OH)_2 intracanal dressing because it is ineffective when used as short-term medicament (Sjögren *et al.* 1991). Although Ca(OH)_2 is a good antimicrobial agent, it is ineffective against some of the microorganisms such as *E. faecalis* (Sjögren *et al.* 1991, Haapasalo and Ørstavik 1987, Heling *et al.* 1992), which is found in the case of persistent root canal infection.

To improve the antimicrobial efficacy of Ca(OH)_2 against *E. faecalis*, the combination of Ca(OH)_2 and 2% CHX gel was used in this study. Gomes *et al.* (2006) demonstrated that Ca(OH)_2 mixed with 2% CHX gel had better antimicrobial activity than Ca(OH)_2 manipulated with sterile water in an *in vitro* study. In addition, Siren *et al.* (2004) showed Ca(OH)_2 was unable to kill *E. faecalis* in the dentine, Ca(OH)_2 combined with CHX effectively disinfected the dentine. Other studies also support that mixture of Ca(OH)_2 and CHX solution is more effective in eliminate *E. faecalis* than Ca(OH)_2 alone (Ercan *et al.* 2006, Zerrella *et al.* 2005, Podbielski *et al.* 2003, Evan *et al.* 2003).

In our study, placement of Ca(OH)_2 /2% CHX gel intracanal dressing for at least 2 weeks rendered 91.7 % of canals bacteria free in teeth with apical periodontitis. This value is consistent with the previous reports (**Figure 8**). Shuping *et al.* (2000) had 92.5% of canals bacteria free when placing for Ca(OH)_2 alone at least 1 week. McGurkin-Smith *et al.* (2005)

had 86% of canals bacteria free after 2 weeks of Ca(OH)_2 alone. Interestingly, unlike those studies, we did not find significant improvement of the root canal disinfection with additional intracanal dressing (**Figure 8**). This does not imply that Ca(OH)_2 /2% CHX gel intracanal dressing is ineffective. Rather it demonstrated that 2% CHX gel is an effective root canal disinfectant.

The purpose of two visit root canal treatment using intracanal dressing (such as Ca(OH)_2) is to predictably control the root canal infection (Trope *et al.* 1991). This is important because the negative culture prior to the root canal filling is related to long term root canal treatment success (Sjögren *et al.* 1997, Byström *et al.* 1987). However, some studies questioned the effectiveness of calcium hydroxide to disinfect the canals, and reported a residual flora in the canals after more than one week of the intracanal dressing. (Reit *et al.* 1999, Peters *et al.* 2002, Kvist *et al.* 2004, Waltimo *et al.* 2005). In some cases, residual bacteria in the canal grew in number even in the presence of calcium hydroxide (Peters *et al.* 2002, Waltimo *et al.* 2005). We had a similar finding in our study. Three samples showed no bacteria growth after root canal preparation with 2% CHX gel but the bacteria were detected after two weeks of Ca(OH)_2 /2% CHX gel intracanal dressing. Ideally, one-visit root canal treatment is desirable if predictable root canal disinfection can be achieved. Our results showed that 2% CHX gel is an effective root canal disinfectant and additional intracanal dressing did not significantly improve the disinfection. Hence, we can speculate that one-visit root canal treatment with 2% CHX gel can be performed without compromising its long term success. Nonetheless, further investigations are required to support this hypothesis.

CHAPTER 6

CONCLUSION

There was a statistically significant difference in bacterial reduction between the initial sample (S1) and the post-instrumentation sample (S2) when 2% CHX gel used as root canal disinfectant. Instrumentation with 2% CHX gel was effective in bacterial reduction and resulted in 89.7 % of canals becoming free of bacteria. The addition of the mixture of Ca(OH)_2 and 2% CHX gel paste as an intracanal medicament for at least two weeks produced 91.7 % of canals void of bacteria. However, there is no statistically significant decrease in the numbers of canal free of bacteria between the post-instrumentation sample (S2) and post intracanal medicament sample (S3).

CHAPTER 7

SUMMARY

In this study, we assessed the ability of Ni-Ti rotary instrumentation with 2% CHX gel in the reduction of intracanal bacteria. In addition, the effect of at least 2 weeks of Ca(OH)_2 /2% CHX gel dressing on intracanal bacteria was also evaluated.

Forty-three patients with clinical and radiographic signs of apical periodontitis were accepted into the study. All patients received instrumentation with 0.04 NiTi rotary instruments and 2% CHX gel, followed by at least 2 weeks of Ca(OH)_2 /2% CHX gel therapy. Four additional vital teeth with a diagnosis of irreversible pulpitis and a normal periapex were treated in the same manner as the study teeth and served as negative controls. The mesiobuccal canals of mandibular molars, distobuccal canals of maxillary molars, and buccal canals of upper premolars were instrumented to apical size #40.04. All single-rooted teeth were instrumented to apical size #60.04. Samples were collected from those canals.

All teeth were sampled prior to (S1), after instrumentation (S2), and after 2 weeks of Ca(OH)_2 /2% CHX gel dressing (S3). The teeth were sampled using the pumping maximum removal method with Liquid Dental Transport Media (LDTM) as the sampling media. All samples were diluted, plated on sheep agar, and incubated anaerobically at 37°C for seven days. The plates were then observed, the CFUs counted, and positive culture counted.

Fisher's exact tests were used to detect the differences in the number of positive culture between S1 and S2, S1 and S3, or S2 and S3. The level of significance was set at 0.05 for all analyses.

The relationship between bacteria and apical periodontitis was shown from the initial samples of the study subjects. Four samples showed no bacteria growth at S1. Of the samples with positive growth (n=39), 10.3% (4/39) sampled bacteria at S2. At S3, three samples were lost during culture and 8.3% (3/36) of the remaining samples cultured positively. All the cases cultured positively at S2 showed no growth at S3. However, three samples cultured negatively at S2 showed bacteria growth at S3. The Fisher's exact test showed a significant difference in the percentage of positive culture between S1 and S2 ($p<0.0001$) and between S1 and S3 ($p<0.0001$). No significant difference was found between S2 and S3 ($p=0.692$).

These results suggest that 2% CHX gel is an effective root canal disinfectant and can be used alternative to NaOCl solution.

Appendix I

Consent Form Example

University of North Carolina-Chapel Hill
Consent to Participate in a Research Study
Adult Subjects
Biomedical Form

IRB Study # 05-DENT-769
Consent Form Version Date: 1-18-2006

Title of Study: Efficacy of 2% Chlorhexidine Gel in Disinfecting the Root-Canal System: a clinical study

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What are some general things you should know about research studies?

You are being asked to take part in a research study. To join the study is voluntary. You may refuse to join, or you may withdraw your consent to be in the study, for any reason.

Research studies are designed to obtain new knowledge that may help other people in the future. You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies.

Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or the University of North Carolina-Chapel Hill. If you are a patient with an illness, you do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study. You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

What is the purpose of this study?

The purpose of this study is to test how effective 2% chlorhexidine gel can disinfect a root canal. To reduce the bacteria in a root canal is important, because it can significantly affect the long term outcome of root canal treatment. Chlorhexidine, an antiseptic belonging to family of biguanides, is used extensively in the medical and surgical environment. In dentistry, it is often used as mouth rinse solution.

Recently, 2% chlorhexidine gel was introduced as root canal disinfectant. Research studies not using patients have shown that 2% chlorhexidine gel is a very effective antimicrobial agent for disinfecting root canal, especially it can kill certain bacteria resisting to the conventional disinfectant. In addition, chlorhexidine does cause obvious toxic effects when used intraorally. Unlike some disinfectant, it does not have bad taste and stain cloth easily. Therefore, these properties make 2% chlorhexidine gel a suitable alternative to the conventional root canal disinfectant.

In this study, we want to evaluate the efficacy of 2% chlorhexidine gel clinically.

You are being asked to be in the study because one of your teeth has been diagnosed as necrotic pulp with apical periodontitis, indicating the need for a root canal treatment.

Are there any reasons you should not be in this study?

Pregnant women, decisionally impaired patients and patients younger than 14 years old will be excluded from the study. Medically compromised patients with conditions that are contraindicated to the dental treatment will also be excluded from the study. These include severe hypertension, uncontrollable diabetes, recent history of stroke, etc

How many people will take part in this study?

If you decide to be in this study, you will be one of approximately 40 people in this research study.

How long will your part in this study last?

You are not expected to spend any more time than 2 regular visits for your root canal treatment.

What will happen if you take part in the study?

You will have a routine root canal treatment (RCT) that follows the UNC endodontic protocol. The exception is that you might be treated with either sodium hypochlorite or 2% chlorhexidine gel as the disinfectant during the treatment. Sodium hypochlorite is most commonly used disinfectant in RCT and this solution will be used as control in this study. You will be randomly assigned to be treated with either 2% chlorhexidine gel or sodium hypochlorite.

During the treatment, we will take bacterial samples from the root canals of your treating tooth. Total of 3 samples at 3 different time period will be taken: 1) before cleaning root canal 2) after cleaning root canal, and 3) right before root canal filling. Bacterial samples will be sent to a microbiologic lab as soon as possible for analysis. The number of bacterial count will be analyzed to examine the effectiveness of 2% chlorhexidine gel in disinfecting root canals compared to that of sodium hypochlorite.

What are the possible benefits from being in this study?

Research is designed to benefit society by gaining new knowledge.

A potential benefit to you from being in this study is that you might have better healing process after the treatment when using 2% chlorhexidine gel as compared to that of sodium hypochlorite.

In addition, you might have less unpleasant experience from foul taste and toxic effects when using 2% chlorhexidine gel as the disinfectant. Although there are possible benefits from being in this study, this cannot be guaranteed.

What are the possible risks or discomforts involved with being in this study?

It is expected that the procedures incur minimal risk. You should be aware that the treatment in this study has the same risks and possible complications as all root canal procedures, including modest pain following treatment and failure for the tooth to completely heal despite treatment. Anytime a root canal treatment is undertaken, modest pain may be anticipated. You will receive a prescription for medication or reduce discomfort if needed, though this is usually unnecessary.

Chlorhexidine is a relatively nontoxic material. Research studies have shown that 2% chlorhexidine solution when used as a periodontal irrigant did not cause obvious toxic effects on gingival tissue, suggesting its safety for intraoral use. In very rare occasion, chlorhexidine can cause acute allergic reaction when applied to skin or mucosa. However, there has been no report of allergic reaction to chlorhexidine when used as the root canal disinfectant.

In addition, there may be uncommon or previously unknown risks that might occur. You should report any problems to the researchers.

What are the risks to a pregnancy or to a nursing child?

Pregnant women will be excluded from this study. We do not know the effect of the study drug on nursing children.

If you choose not to be in the study, what other treatment options do you have?

You do not have to be in this research study in order to receive treatment. The other procedures or treatments that are available include routine root canal treatment.

What if we learn about new findings or information during the study?

You will be given any new information gained during the course of the study that might affect your willingness to continue your participation.

How will your privacy be protected?

Only the treating dentist will know about your identity throughout the study. Specific ID numbers will be given to patients and bacterial samples. Only ID numbers will be used to communicate with the laboratory. At the end of data collection, the patient list from this study will be destroyed by paper shredder.

No subjects will be identified in any report or publication about this study. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research study could be reviewed by representatives of the University, research sponsors, or government agencies for purposes such as quality control or safety.

A copy of this consent form will go in to your medical record. This will allow the doctors caring for you to know what study medications or tests you may be receiving as a part of the study and know how to take care of you if you have other health problems or needs during the study.

What will happen if you are injured by this research?

All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. However, by signing this form, you do not give up any of your legal rights.

What if you want to stop before your part in the study is complete?

You can withdraw from this study at any time, without penalty. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, or have failed to follow instructions, or because the entire study has been stopped.

Will you receive anything for being in this study?

You will not receive anything for taking part in this study.

Will it cost you anything to be in this study?

It will not cost you anything in addition to what you will be billed for your routine root canal treatment to be in this study. All tests, visits or procedures other than what is done for this study will be related to dental care that is part of the usual care for your condition and would be suggested even if you decided not to be in the research study.

What if you are a UNC student?

You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at UNC-Chapel Hill. You will not be offered or receive any special consideration if you take part in this research.

What if you are a UNC employee?

Taking part in this research is not a part of your University duties, and refusing will not affect your job. You will not be offered or receive any special job-related consideration if you take part in this research.

Who is sponsoring this study?

The study is carried out by the Department of Endodontics, UNC School of Dentistry. Currently it is not sponsored by any organization. Investigators have no financial affiliation with the products used in this study.

What if you have questions about this study?

You have the right to ask, and have answered, any questions you may have about this research. If you have questions, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

What if you have questions about your rights as a research subject?

All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject you may contact, anonymously if you wish, the Institutional Review Board at 919-966-3113 or by email to IRB_subjects@unc.edu.

Subject's Agreement:

I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

Signature of Research Subject

Date

Printed Name of Research Subject

Signature of Person Obtaining Consent

Date

Printed Name of Person Obtaining Consent

Table 1. Description of samples

S1: Initial, pre-instrumentation sample

S2: post-instrumentation sample

S3: post-dressing sample

Table 2. Apical preparation size corresponding to canal and tooth type.

Tooth type	Canal	Apical sizes	Taper
Incisors	central	#60	.04
Single rooted premolars	central	#60	.04
Maxillary premolar	buccal	#40	.04
Maxillary molar	disto-buccal	#40	.04
Mandibular molar	mesio-buccal	#40	.04

Table 3. Bacterial counts (CFU/ml) for each sample

Sample#	Tooth#	Apical Size	S1	S2	S3
1	13	60	>10 ⁹	0	0
2	7	60	9.63x10 ⁵	0	**
3	8	60	2.70x10 ⁶	4.87x10 ²	0
4	31	40	6.09x10 ²	0	0
5	7	60	7.50x10 ⁴	0	0
6	31	40	5.50x10 ³	0	0
7	19	40	>10 ⁹	2.84x10 ³	0
8	8	60	1.74x10 ⁵	0	0
9	9	60	1.33x10 ⁵	0	0
10	23	60	1.81x10 ⁵	0	1.01x10 ²
11	30	40	2.00x10 ⁵	0	0
12	4	60	0*	0*	0*
13	19	40	1.71x10 ⁵	0	0
14	10	60	0*	0*	0*
15	14	40	0*	0*	4.80x10 ⁴ *
16	30	40	6.90x10 ⁵	0	0
17	28	60	6.40x10 ²	0	0
18	19	40	5.80x10 ⁵	0	0
19	19	40	2.40x10 ⁷	1.40x10 ⁴	0
20	4	60	4.60x10 ⁴	0	0
21	13	60	1.40x10 ⁷	0	8.90x10 ⁴
22	9	60	2.10x10 ⁷	0	0
23	5	40	1.10x10 ⁷	0	0
24	29	60	0*	0*	0*
25	30	40	>10 ⁹	0	0
26	15	40	>10 ⁹	0	3.20x10 ²
27	14	40	2.50x10 ⁵	0	**
28	19	40	5.50x10 ⁵	0	**
29	7	60	3.10x10 ⁵	1.30x10 ³	0
30	3	40	2.50x10 ⁵	0	0
31	20	60	1.20x10 ⁴	0	0
32	29	60	1.40x10 ⁵	0	0
33	3	40	4.00x10 ³	0	0
34	3	40	2.30x10 ⁴	0	0
35	3	40	8.13x10 ²	0	0
36	9	60	6.00x10 ⁵	0	0
37	23	60	1.00x10 ³	0	0
38	18	40	1.40x10 ⁵	0	0
39	29	60	>10 ⁹	0	0
40	19	40	4.20x10 ⁵	0	0
41	29	60	1.70x10 ⁵	0	0
42	18	40	2.40x10 ⁴	0	0
43	31	40	8.10x10 ⁴	0	0

*Initial samples with no bacterial growth, excluded from analysis

**samples lost during culturing

>10⁹ represents the samples that were too numerous to count

0 represents samples below the limit of detection (<10²)

Table 4. Percentage of samples with negative culture

Groups	Sample Size	S2	S3
Size #60	18	16/18 (88.9%)	15/17 (88.2%)
Size #40	21	19/21 (90.5%)	18/19 (94.7%)
Total	39	35/39 (89.7%)	33/36 (91.7%)

Figure 1: Initial, Pre-Instrumentation Sample (S1)

Figure 2: Post-Instrumentation Sample (S2)

Figure 3: Post-Dressing Sample (S3)

The sample distribution at S1

Figure 4. The sample distribution at S1. x represents the number of bacterial count (CFU/ml). $x < 10^2$ represents the samples that were below the limit of detection and considered as the negative culture. $x > 10^9$ represents the samples that were too numerous to count.

The sample distribution at S2

Figure 5. The sample distribution at S2. x represents the number of bacterial count (CFU/ml). $x < 10^2$ represents the samples that were below the limit of detection and considered as the negative culture. $x > 10^9$ represents the samples that were too numerous to count

Figure 6. The sample distribution at S3. x represents the number of bacterial count (CFU/ml). $x < 10^2$ represents the samples that were below the limit of detection and considered as the negative culture. $x > 10^9$ represents the samples that were too numerous to count

Figure 7. Percentage of samples with positive culture

Figure 8. Comparison of percentage of canals with positive culture at different culturing points from selected studies

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