**THE IN VIVO ANTIBACTERIAL EFFICACY OF ULTRASOUND
AFTER HAND AND ROTARY INSTRUMENTATION IN HUMAN
MANDIBULAR MOLARS**

A Thesis

Presented in Partial Fulfillment of the Requirements for
the degree of Master of Science in the
Graduate School of The Ohio State University

By

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2005

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ABSTRACT

The use of ultrasound as an adjunct to conventional instrumentation procedures has been suggested to enhance the removal of bacteria and necrotic debris from infected root canals. The purpose of this in vivo, prospective, randomized, blinded study was to compare the antibacterial efficacy, by means of microbial culture methods and the specific ability to eliminate *E. faecalis* as measured by polymerase chain reaction (PCR), of a hand and rotary instrumentation technique versus a hand and rotary instrumentation plus one-minute ultrasound technique using an ultrasonic irrigating needle connected to a MiniEndo™ piezoelectric ultrasonic system in the mesial roots of infected, necrotic, human mandibular molars. Thirty-three subjects participated in this study. Group 1 consisted of 17 mesial roots prepared with a hand and rotary instrumentation technique and conventional irrigation with 6.0% sodium hypochlorite. Group 2 consisted of 16 mesial roots prepared in a similar manner followed by 1 minute of ultrasonic irrigation with 6.0% sodium hypochlorite per canal. The canals were sampled prior to treatment, after instrumentation for both groups, and after syringe irrigation for teeth in Group 1 and after ultrasonic irrigation for teeth in Group 2. The samples were incubated anaerobically for 7 days at 37°C, the bacteria from each sample were quantified, and the CFU counts and log₁₀ CFU counts were used for statistical analysis. Samples displaying initial

growth and growth after instrumentation or ultrasonic irrigation were submitted for PCR detection of *E. faecalis*.

All samples were positive for initial growth. Statistical analysis using the Exact Mann-Whitney-Wilcoxon test indicated no significant differences between Group 1 and Group 2 with regard to initial ($p = 0.385$) or post-instrumentation ($p = 0.093$) CFU counts. A dependent t-test showed hand and rotary instrumentation with 6.0% sodium hypochlorite irrigation significantly ($p < 0.0001$) reduced bacterial numbers from $\log_{10}$ initial CFU counts in each group. Nonparametric analysis using Chi-Square revealed the addition of ultrasonic irrigation in Group 2 resulted in significantly ($p = 0.038$) more canals that cultured no bacteria. Logistic regression analysis indicated the addition of ultrasonic irrigation was 6.98 times more likely to yield a negative culture. The results of this study indicated the addition of ultrasonic irrigation after hand and rotary instrumentation increases the likelihood of obtaining a negative culture in the mesial roots of infected, necrotic, human mandibular molars.

To Leslie, with all my love, devotion, and appreciation.
I love you (more).

To my parents and my sisters, for always being there and standing
beside me even through the toughest of times.

To John, for introducing me to dentistry and all the wonderful
opportunities it beholds.

ACKNOWLEDGMENTS

I wish to thank my advisor, **Dr. John Nusstein**, whose vision and support made this thesis possible. Thank you for your guidance, for teaching me common-sense endodontics, and for always listening.

I thank **Dr. Al Reader** for an educational experience second to none. Thank you for stimulating in me a life-long commitment to learning. I am forever grateful.

I thank **Dr. William Meyers** for his dedication to endodontics, and for many conversations without a clear winner. Congratulations on all of your accomplishments.

I thank my fellow residents, **Dr. Louis Susi**, **Dr. Joanne Jensen**, and **Dr. Vaishali Agarwala**. I have greatly enjoyed the past two years and the friendships we have forged. Completing the program would not have been possible, and certainly not as enjoyable, without each one of you.

I would like to thank **Dr. Michael Beck** for his statistical interpretation of the data.

I thank **Jeff Zelli**, whose knowledge of computers and word processing allowed me to complete this manuscript.

I would also like to thank **Dr. Gene Leys**, **Christina Igboin**, and **Kashmira Asnani** for the use of their laboratory and help with the microbiology and PCR procedures.

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CHAPTER 1

INTRODUCTION

Endodontic success relies on thorough chemomechanical preparation of the root canal system (1-3). Adequate canal debridement is necessary to eliminate potential contaminants such as microorganisms, microbial by-products, and pulp tissue from the root canal system. Failure to eliminate these irritants from the root canal system is recognized as a primary reason for failure of endodontic therapy (4).

The intricate nature of root canal anatomy complicates the instrumentation procedure (5-13). Fins, webs, anastomoses, isthmuses and other irregularities within the root canal system harbor tissue, microorganisms, and microbial by-products that may lead to failure of root canal therapy (9-11). These areas were demonstrated to be inaccessible to conventional hand and rotary instrumentation (15-27).

In vivo research has failed to demonstrate complete elimination of the microbial population within necrotic molar root canals following hand and/or rotary instrumentation. Byström and Sundqvist (28) found a 100- to 1000-fold reduction in bacterial numbers following hand instrumentation and saline irrigation, yet no teeth cultured free of bacteria. Dalton et al. (29) found significant bacterial reduction following rotary instrumentation and irrigation with saline; however, only 28% of the teeth sampled were free of bacteria. Shuping et al. (30) attained negative cultures in only

62% of teeth following nickel-titanium rotary instrumentation and irrigation with 1.25% NaOCl. This number increased to 92% following 1 week of calcium hydroxide therapy. Card et al. (31) eliminated 93% of intracanal bacteria in molar mesial canals in a study analyzing the effectiveness of increased apical enlargement with 1% NaOCl irrigation in reducing intracanal bacteria. The number of remaining bacteria following root canal therapy that could lead to failure of endodontic treatment is unknown; therefore, improving the reduction of viable bacteria within the root canal system should, empirically, improve the success rate. More effective instrumentation techniques are required in order to eliminate as many, if not all, bacteria and bacterial by-products from the root canal system.

The use of ultrasonics provides a possible solution to the problem of debriding and disinfecting the intricate root canal system. Martin and Cunningham et al. (32-38) found that teeth instrumented ultrasonically retained less debris than teeth instrumented by hand. However, other authors (39-44) have not demonstrated superior tissue removal results when comparing ultrasound as a primary method of instrumentation to hand instrumentation. Cymerman et al. (40) determined both hand and ultrasonic instrumentation left statistically equal amounts of tissue debris in the canals when viewed by scanning electron microscopy (SEM). Pedicord et al. (42) found hand instrumentation was faster and produced better-shaped canals than ultrasonic instrumentation. Reynolds et al. (43) found hand instrumentation was superior to sonic and ultrasonic instrumentation in increasing canal size, removing predentin and tissue debris, and planing the canal walls.

Researchers have investigated the mechanisms of ultrasonic instrumentation to determine why the early successes of ultrasonic instrumentation could not be reproduced. In a series of investigations, Ahmad et al. (45-48) concluded that dampening (file contacting the walls of the canal) of the vibrating file in the confines of a root canal prevents the production of effective ultrasonic energy. Walmsley (49) also concluded that propagation of ultrasonic waves is inhibited by inadequate root canal space that restricts free vibration of the energized file.

Past research also investigated ultrasound as an adjunct to conventional hand instrumentation instead of a primary cleaning method (14-18). The use of an ultrasonic technique after completion of hand instrumentation should reduce the constriction of the vibrating file inside the root canal space. Studying the use of ultrasound after hand instrumentation, Weller et al. (14) found that serialized hand preparation and the addition of ultrasound was more effective in cleaning canals than either method alone. Goodman et al. (15) used a compensating polar planimeter to compare the tissue removing capability of a step-back/ultrasound method. Using extracted mandibular molars, the authors found the combination method was more effective in removing tissue at the 1 mm and 3 mm levels as well as the isthmuses. However, this study utilized a modified piezoelectric ultrasonic dental unit that was not commercially available.

Using the same method to measure tissue removal as Goodman et al. (15), Lev et al. (16) compared a step-back/ultrasound technique using the Cavitron® Cavi-Endo® ultrasonic unit for 1 and 3 minutes versus a step-back technique alone. Canal debridement did not significantly vary among any of the groups. The three-minute step-back/ultrasound group had significantly cleaner isthmuses at both the 1 mm and 3 mm

apical levels than the other groups. Haidet et al. (17) used the Cavitron® Cavi-Endo® dental unit to histologically compare the tissue removal of step-back versus step-back/ultrasound instrumentation in the mesial roots of mandibular molars. This in vivo study used the same method of tissue removal evaluation as Goodman et al. (15) and evaluated the 1 mm and 3 mm apical levels along with isthmuses. At the 1 mm level, canals and isthmuses were both significantly cleaner with the combination method. At the 3 mm level, no significant difference was found between the two methods.

Metzler and Montgomery (18) compared the effectiveness of ultrasonics using a Cavitron® dental unit with a Cavi-Endo® insert and calcium hydroxide for the debridement of extracted human mandibular molars. The Cavitron® unit was used at a higher frequency setting than the Cavi-Endo® unit noted in previous studies (16,17). In the first group, ultrasound was used for 2 minutes following step-back hand instrumentation. In the second group, calcium hydroxide was placed in the canal immediately following hand instrumentation and remained for 1 week. The Olympus CUE-2 Image Analysis program evaluated tissue removal from the canals. The results showed ultrasonics and calcium hydroxide were equally effective in debriding the root canal system, and both methods were significantly better than hand instrumentation alone in the isthmuses at the 1 mm apical level.

The Cavi-Endo® and Cavitron® systems used in the previously mentioned studies (16-18) are limited in their output of ultrasonic energy due to the use of a magnetostrictor as an ultrasonic source (50). Stamos et al. (51) compared the debridement efficacy of the Cavi-Endo® system to the more powerful Osada Enac piezoelectric ultrasonic system. The Enac system cleaned a significantly higher percentage of the canal space when

compared to the Cavi-Endo[®] system. Ahmad et al. (45-47) examined the mechanisms of ultrasonic energy involved in canal debridement using the same two ultrasonic systems. Using a photometric detection system, the authors determined the Cavi-Endo[®] unit was unable to produce cavitation (the growth and implosion of gas bubbles). Instead, the limited ultrasonic energy generated by the Cavi-Endo[®] unit produced only acoustic streaming (vortex movement of fluid particles around the vibrating file). The Enac unit, however, was capable of producing cavitation due to an increased output of ultrasonic energy. In an in vitro study with extracted, single-rooted teeth, Ahmad et al. (45) found no difference in smear layer removal between the groups with and without cavitation. However, the authors did not evaluate the tissue removing capability of the two systems. These investigators also determined the ultrasonic energy output of the Enac system was limited when used as a primary form of instrumentation, and cavitation could not be produced when the vibrating file was dampened in a small canal. Roy et al. (52) demonstrated transient cavitation and acoustic streaming using a Piezon[®] - Master 400 piezoelectric ultrasonic system; however, this study did not evaluate the effects of ultrasound on tissue debridement.

Siqueira et al. (53) found no significant differences in apical third canal cleanliness when comparing the Enac system (#15 file/1 minute/no irrigation) to step-back instrumentation with stainless steel files, step-back instrumentation with nickel-titanium files, and the balanced forces instrumentation technique. However, the authors only prepared the canals to a size #15 file before the Enac system was used. Heard et al. (54) also found no significant differences in the apical, middle, and coronal thirds when

the Enac system (step-back, flaring, and ultrasound with H₂O/NaOCl irrigation) was compared to step-back instrumentation with and without flaring and ultrasound alone.

Archer et al. (27) used the Osada Enac piezoelectric ultrasonic system to histologically compare tissue removal of step-back instrumentation versus step-back plus ultrasound instrumentation in the mesial roots of mandibular molars. This study utilized an Olympus CUE-2 Image Analysis program to evaluate tissue removal at the 1, 2, and 3 mm apical levels of canals and isthmuses. Canal and isthmus cleanliness values were significantly higher at all apical levels evaluated for the step-back/ultrasound group.

Past research has also evaluated the antibacterial effect of ultrasound as a primary instrumentation method and following hand instrumentation. Sjögren and Sundqvist (55) determined that ultrasonics with 0.5% NaOCl eliminated bacteria more efficiently than hand instrumentation alone in single-rooted necrotic teeth with periapical areas. Yoshida et al. (56) eliminated bacteria from 93 of 129 necrotic root canals using a 15% EDTA solution with ultrasonic agitation. Using passive ultrasonic activation with saline irrigation in an in vitro model, Spoleti et al. (57) found significantly less bacterial colonies in groups treated with ultrasonics. Ahmad et al. (58) demonstrated decreased counts of *S. mitis* in extracted teeth treated with the Osada Enac ultrasonic system and 2.5% NaOCl when compared to teeth treated with ultrasonics alone. Huque et al. (59) found that ultrasonic irrigation with 12% NaOCl eliminated bacterial cultures from surface, shallow, and deep layers of root dentin in extracted teeth more efficiently than ultrasonic irrigation with 5.5% NaOCl, 15% EDTA, or saline. Martin (32) determined ultrasonic energy alone was capable of reducing bacterial numbers but was more effective when combined with a biocidal agent. Conversely, Siqueira et al. (60) failed to

demonstrate a significant difference in reducing intracanal numbers of *E. faecalis* when teeth were treated by irrigation with 2 mL of 4% NaOCl solution and agitation with hand files, irrigation with 2 mL of 4% NaOCl solution and ultrasonic agitation, or irrigation with 4% NaOCl alternated with hydrogen peroxide.

No study to date has examined the antibacterial efficacy of an ultrasonic irrigating needle as an adjunct to hand/rotary instrumentation. The needle, when connected to a MiniEndo™ piezoelectric ultrasonic system, was reported to have high ultrasonic output and to produce cavitation in an instrumented canal (61). This more powerful unit is capable of producing a faster rate of acoustic streaming, and was shown to debride canal systems significantly better than hand/rotary filing alone (61). Gutarts et al. (62) examined the in vivo debridement efficacy of an ultrasonic irrigating needle activated for 1 minute as an adjunct to hand/rotary instrumentation and determined that less dentinal debris and pulp tissue were found using the ultrasonic irrigating needle regardless of canal type, canal curvature, or apical level. Studies by Goodman et al. (15), Lev et al. (16), and Haidet et al. (17) using ultrasonic files after canal preparation utilized either additional volumes of irrigating solution or filing after the step-back/ultrasound procedure to achieve their results. While these additions were needed to control the variables of ultrasonic instrumentation, these procedures are not generally performed clinically. These studies, as well as the study by Archer et al. (27), utilized a three-minute ultrasonic cleaning cycle per canal using endodontic file inserts. These methods resulted in file breakage when subjected to the higher range of ultrasonic energy. Therefore, the files were energized at low settings resulting in an inordinate amount of time to clean the root canals. By using a disposable, irrigating needle that can withstand

the higher range of energy settings, the cleaning time was reduced from 3 minutes to 1 minute per canal. A one-minute cycle per canal may be more clinically acceptable for endodontic treatment. Additionally, the sodium hypochlorite irrigating solution can be delivered directly through the needle at a controlled rate. This method of delivering irrigating solution offers an advantage over previous methods where the solution was added at the coronal location using a separate syringe.

Therefore, the purpose of this in vivo, prospective, randomized, blinded study was to compare the antibacterial efficacy, by means of microbial culture methods and the specific ability to eliminate *E. faecalis* as measured by polymerase chain reaction (PCR), of a hand and rotary instrumentation technique versus a hand and rotary instrumentation plus one-minute ultrasound technique using an ultrasonic irrigating needle connected to a MiniEndo™ piezoelectric ultrasonic system in the mesial roots of infected, necrotic, human mandibular molars.

CHAPTER 2

LITERATURE REVIEW

Selected portions of the following Literature Review were adapted from previous theses by Gutarts et al. (62), Goodman et al. (260), Lev et al. (261), Haidet et al. (262), and Archer et al. (263) from the Department of Endodontics at The Ohio State University.

MICROBIOLOGY OF THE ROOT CANAL AND PERIRADICULAR TISSUES

A number of studies (63-66) have established the role of bacteria in pulpal and periradicular pathosis. In 1894, Miller (63) first demonstrated the presence of bacteria in a study of more than 250 necrotic dental pulps. The author suggested many microorganisms were uncultivable due to the presence of competing species, and observed the presence of spirochetes as well as the fact that some bacteria were more virulent when experimental infections were made in animals.

Kakehashi, Stanley, and Fitzgerald (64) established the role of bacteria in pulpal and periradicular pathosis using germ-free and conventional laboratory rats. The authors created pulp exposures in 21 germ-free and 15 conventional laboratory rats and made no attempt to restore or seal the exposure site. The effects on pulpal and periradicular tissues were histologically analyzed after 1 to 42 days. Pulpal necrosis with chronic

inflammatory tissue and abscess formation was noted in the tissues of the conventional rats. The injured pulpal tissues of this group showed no evidence of repair. The pulpal inflammation resulting from the exposure in germ-free rats was minimal in every specimen. No completely devitalized pulps were found in any specimen and no abscess formation was noted. Dentinal bridging was evident at 14 days, with vital pulpal tissue present beneath the newly formed dentinal bridge. The authors concluded that viable microorganisms are requisite for pulpal and periradicular inflammation.

Möller et al. (65) histologically and microbiologically examined the necrotic pulps of monkey teeth either kept bacteria-free by sealing or infected by the indigenous oral flora. The results were recorded at the beginning of the experiment and after 6 to 7 months. The authors found the non-infected necrotic pulp tissue did not induce inflammatory reactions in the apical tissues. Teeth infected by the indigenous oral flora showed strong periapical inflammatory reactions histologically. Obligate anaerobes dominated the microbial flora within the infected root canal. The authors concluded that bacteria are essential for the pathogenesis of apical periodontitis.

Fabricius et al. (66) analyzed the influence of polymicrobial infections on the periapical tissues of monkeys. Combinations of 11 strains of bacteria were inoculated together in a total of 75 root canals. The authors found that *Bacteroides oralis* predominated in mixed infections but was not re-isolated when inoculated in pure culture, suggesting the importance of bacterial synergism in perpetuating infection. Enterococci strains were recoverable as pure specimens. Periapical tissue reactions were more likely to occur with mixed bacterial infections. Gomes et al. (67) and Sundqvist (68) also noted bacterial species within the root canal have a tendency to associate together. This may be

due to the production of essential metabolic by-products by one species for another species (68-69) or by selective inhibition from the production of bacteriocins (70).

These studies collectively demonstrated that bacteria play an important role in pulpal and periradicular pathosis, and suggested that bacterial synergism is integral in perpetuating infection.

The Microbial Flora of the Necrotic Dental Pulp

Kobayashi et al. (71) established the source of bacteria in root canal infections. This in vivo study compared the microbial flora of necrotic teeth affected with advanced periodontitis using anaerobic culturing techniques and interference microscopy. The authors found a greater predominance of anaerobes in the necrotic dental pulp than in the periodontal pocket reflecting the largely anaerobic environment found within the root canal. Bacterial species common to both sites included *Streptococcus*, *Peptostreptococcus*, *Eubacterium*, and *Bacteroides*. Similar proportions of bacterial morphotypes were found in the periodontal pocket and in the root canal, suggesting the gingival sulcus may be a source of bacteria in root canal infections.

Fabricius et al. (72) described the predominant bacteria isolated from infected root canals of monkeys after 7, 90, 180, and 1060 days of closure. The teeth were initially devitalized, left exposed to the oral flora for 7 days, and then sealed. The authors found that 85% to 98% of the bacterial strains cultivated were anaerobic. The most frequently encountered species were *Bacteroides* and Gram-positive anaerobic rods.

Baumgartner et al. (73) demonstrated the predominance of anaerobic bacteria in infected human root canals. The apical 5 mm of 10 freshly extracted teeth with carious pulpal exposures and periapical lesions contiguous with the root apex were cultured

aerobically and anaerobically. The most prominent species found included members of *Actinomyces*, *Lactobacillus*, black-pigmented *Bacteroides*, *Peptostreptococcus*, non-pigmented *Bacteroides*, *Veillonella*, *Enterococcus faecalis*, *Fusobacterium nucleatum*, and *Streptococcus mutans*. Sixty-eight percent of the 50 bacterial isolates cultured were anaerobic, demonstrating the high proportion of these bacteria in necrotic dental pulps.

Sundqvist (74) described the microbial population isolated from the root canals of human teeth with apical periodontitis. In decreasing order of incidence, these microorganisms included *F. nucleatum*, *Streptococcus* spp., *Bacteroides* spp., *P. intermedia*, *P. micros*, *P. alactolyticus*, *P. anaerobius*, *Lactobacillus* spp., *E. lenta*, *Fusobacterium* spp., *Campylobacter* spp., *Peptostreptococcus* spp., *Actinomyces* spp., *M. timidum*, *C. achracea*, *E. brachy*, *S. sputigena*, *V. parvula*, *P. endodontalis*, *P. buccae*, *P. oralis*, *P. propionicum*, *P. denticola*, *P. loescheii*, and *E. nodatum*. The author concluded obligate anaerobes dominate the microbial flora of human infected root canals.

Kantz and Henry (75) and Wittgow and Sabiston (76) further demonstrated the prevalence of obligate anaerobes in the root canals of infected human teeth. Kantz and Henry (75) cultured 24 necrotic pulps with intact pulp chambers from 20 patients. Nitrogen gas was streamed over the tooth surfaces during sample acquisition to help maintain an anaerobic environment. Direct microscopic clump counts (DMCC) were obtained and individual bacterial species were identified using selective media. The DMCC ranged from 8.5×10^5 to 3.8×10^8 per mL of sample with a mean of 3.3×10^7 per mL of sample. Viable anaerobic bacteria recovery counts ranged from 0 to 1.5×10^7 per mL of sample, representing 45% of the total DMCC. The anaerobes isolated included *Actinomyces israelii*, *B. fragilis*, *B. melaninogenicus*, *C. sputorum*, *E. alactolyticum*, *F.*

fusiforme, *F. rarium*, *P. morbillorum*, *P. acnes*, and *V. parvula*. Wittgow and Sabiston (76) cultured 40 intact teeth from 33 patients with necrotic pulps due to trauma. All teeth cultured were incisors. Thirty-six of the teeth had necrotic pulp chambers and 30 of these had periapical radiolucencies. Thirty-two of 40 teeth yielded positive cultures, and 97% of these harbored obligate anaerobes. A total of 82 species were identified, most of which were part of the normal oral flora. Anaerobic gram-negative rods were found in 67% of all teeth sampled, in 75% of all teeth with necrotic pulps, and in 84% of all teeth with positive cultures. Three or more different species of microorganisms were found in 50% of teeth with positive cultures. The authors concluded that caution should be taken during instrumentation in order to prevent over-instrumentation and subsequent injection of these microorganisms into the alveolar bone, which may result in acute flare-ups occasionally associated with endodontic therapy on asymptomatic teeth.

Drucker et al. (77) showed the presence of obligate anaerobes in 45% of specimens cultured from 35 infected root canals. Forty different species were isolated, with the most common being *Streptococcus sanguis* and *Peptostreptococcus micros*. *Prevotella melaninogenica*, *Enterococcus faecalis*, and *Prevotella oralis* were isolated in 14% of specimens. Significant associations were found between *P. melaninogenica* and *P. micros*, *P. melaninogenica* and *P. oralis*, *Prevotella corporis* and *Streptococcus morbillorum*, and *Actinomyces odontolyticus* and *E. faecalis*.

Past research (78-80) identified *P. nigrescens* as the most common black-pigmented bacterium in endodontic infections. Bae et al. (78) determined the occurrence of *P. nigrescens* and *P. intermedia* in root canal infections used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A unique 18.6 kDa protein band was

used to distinguish between the two species. The authors identified 56 strains of black-pigmented bacteria, with *P. nigrescens* representing 73% and *P. intermedia* 27% of the total strains present. The authors concluded that *P. nigrescens*, and not *P. intermedia*, is the most common black-pigmented bacterium in endodontic infections.

Gharbia et al. (79) also utilized SDS-PAGE of unique whole cell proteins to determine the incidence of *P. nigrescens* and *P. intermedia* in 73 strains tentatively identified as either *P. nigrescens* or *P. intermedia* isolated from necrotic root canals and periodontal sites. *Prevotella intermedia* comprised 29 of 41 strains from periodontal sites, and only 4 of 15 strains from necrotic root canals. The authors concluded site specificity may be an important determinant in the pathogenicity of *P. nigrescens* and *P. intermedia*.

Dougherty et al. (80) cultured the coronal and apical segments from 18 necrotic root canals with associated periapical radiolucencies in an effort to identify the ecological relationships between strains of black-pigmented bacteria. Twelve sampled roots were positive for the presence of black-pigmented bacteria, and 8 of these 12 roots had a carious pulp exposure. *Prevotella nigrescens* was identified in the coronal and apical segments of 7 teeth, and was identified in all teeth with growth of black-pigmented bacteria.

Xia et al. (81) identified the presence of the black-pigmented bacterium *Prevotella tanneriae* in 118 samples from necrotic root canals. Using polymerase chain reaction (PCR) analysis, *P. tanneriae* was found in 60% of the samples, indicating that bacteria not routinely cultivable from endodontic infections may contribute to the pathogenicity of apical periodontitis.

Porphyromonas endodontalis has also been isolated from the root canals of symptomatic and asymptomatic teeth. De Oliveira et al. (82) utilized 16S rRNA gene-directed PCR to detect the presence of *P. endodontalis* in 43 infected root canals. *Porphyromonas endodontalis* was detected in 67% of the cases with an acute periradicular abscess, in 53% of the cases exhibiting tenderness to percussion, and in 25% of the cases with an asymptomatic periradicular lesion. In a review of the role of *P. endodontalis* in endodontic infections, van Winkelhoff et al. (83) stated a strain of this microorganism has a capsule that confers resistance to phagocytosis, serum sensitivity, and enhanced pathogenicity. In addition, several toxic metabolic by-products are produced, including butyrate and propionate, which are inhibitors of various cultured human cell lines. Furthermore, Kilian (84) determined that *P. endodontalis* was capable of degrading IgG, IgM, IgA₁, IgA₂, C3, C5, and haptoglobin. The virulence factors of *P. endodontalis* designate its role in symptomatic infections of endodontic origin.

Several authors (85-88) analyzed the relationship of mixed anaerobic infections to the severity of infection. Siqueira et al. (85) illustrated the polymicrobial nature of the root canal microflora using checkerboard DNA-DNA hybridization for detection of 42 bacterial species in 28 sampled root canals. The authors found an average of 5 bacterial species in the root canals sampled. The most common isolates were *B. forsythus*, *H. aphrophilus*, *C. matruchotii*, *P. gingivalis*, and *T. denticola*.

Baumgartner et al. (86) observed that a mixed culture of *F. nucleatum* with either *P. gingivalis* or *P. intermedia* was significantly more pathogenic than a pure culture of *F. nucleatum* alone in producing subcutaneous abscesses in mice.

Price et al. (87) injected CD-1 mice intra-peritoneally with *B. intermedius*, *F. necrophorum* or mixtures of both organisms in an effort to characterize bacterial synergy. Abscess formation did not occur when *B. intermedius* was injected alone, whereas abscess formation occurred in 1 to 2 weeks following injection of the mixed culture. The authors observed that both species persisted in tissue for 22 weeks following mixed infection and concluded the persistence of *B. intermedius* in tissue represents a synergistic relationship with *F. necrophorum* and contributes to the timing of abdominal abscess formation.

Sundqvist et al. (88) tested the ability of combinations of bacteria isolated from infected root canals exhibiting periapical bone destruction to induce subcutaneous abscess formation in guinea pigs. Abscess formation was induced only with combinations of bacteria isolated from teeth with purulent apical inflammation. *Bacteroides melaninogenicus* and *B. asaccharolyticus* were contained in all combinations that induced abscess formation. The authors stated that additional microorganisms, such as *P. micros*, were also required to achieve pathogenicity.

The Microbial Flora of Periradicular Lesions

No consensus in the literature has been reached concerning the presence of bacteria in periapical lesions. Iwu et al. (89) studied 16 periapical granulomas and demonstrated 88% positive growth of bacteria in culture. Forty-seven total isolates were obtained, with 55% representing facultative anaerobes and 45% representing obligate anaerobes. The most common organisms present were *Veillonella* spp., *S. millieri*, *S. sanguis*, *A. naeslundii*, *P. acnes*, and *Bacteroides* species.

Abou-Rass et al. (90) investigated 13 periapical lesions harvested from cases involving refractory endodontic therapy or pulpal calcification. The authors demonstrated the presence of microorganisms in all 13 cases, with 64% of the bacteria representing obligate anaerobes and 36% representing facultative anaerobes. The most commonly isolated organisms included *Actinomyces* spp., *Propionobacterium* spp., *Streptococcus* spp., *Staphylococcus* spp., *P. gingivalis*, *P. micros* and Gram-negative enterics. The authors concluded the persistence of microorganisms may be a determining factor in failed endodontic cases.

Wayman et al. (91) histologically and microbiologically analyzed 58 endodontic cases requiring periapical surgery. Fifty-one out of 58 cultures were positive for bacteria, while bacteria were seen histologically in only 8 of 58 cases. Fifty different species of bacteria were cultured, with 133 isolates identified. Bacteria were present in periapical granulomas, periapical abscesses, and radicular cysts. The authors concluded that bacteria, along with other particulate matter, may contribute to non-healing periapical lesions.

Gatti et al. (92) utilized DNA-DNA hybridization to detect the presence of microorganisms in 36 asymptomatic periapical lesions. Bacteria were detected in all 36 periapical lesions studied. Patients with a sinus tract, teeth lacking an intact full-coverage crown, and teeth with a history of trauma demonstrated a tendency for more genomic variation. Only *B. forsythus* and *A. naeslundii* genospecies 2 were present in large numbers. The authors concluded the presence of bacteria may lead to the persistence of periapical lesions.

Walton and Ardjmand (93) evaluated histologically the presence of bacteria in 18 induced periapical lesions in squirrel monkeys. Pulp were exposed, left open to the oral cavity for 1 week, and then sealed with amalgam. Periapical inflammatory lesions were present radiographically after a period of 7 months. The monkeys were sacrificed and block sections were removed to include the tooth, half of the adjacent teeth, and surrounding bone in order to retrieve the entire periapical lesion. The presence of bacteria on the coronal tooth surface and in the canal space and periapex was then determined. Bacteria were present in all of the experimental teeth with necrotic pulps. In 16 of 18 canals, bacteria were confined to the canal space and did not extend to the apical foramen. No colonies of bacteria were found beyond the apical foramen in any of the specimens. The authors concluded the immune response generated in the periapical tissues is sufficient to limit the presence of bacteria beyond the confines of the root canal. Andreason and Rud (94), Block et al. (95), and Langeland et al. (96) also did not consistently demonstrate the presence of bacteria in surgical biopsy samples from periapical lesions.

Viruses and fungi have also been implicated in the pathogenesis of periapical pathosis. Sabeti and Slots (97) investigated the occurrence of human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and herpes simplex virus in microbial samples collected from 34 periapical lesions during periapical surgery. The presence of bacterial co-infection was also determined. Following surface disinfection of the teeth, gingiva, and mucosa with chlorhexidine, a full-thickness mucoperiosteal flap was elevated to expose the periapical lesion area. Bacterial specimens were obtained using sterile paper points placed within the bony crypt for 30 seconds. Viral specimens were obtained using

sterile curettes. Viral transcripts were identified using reverse-transcription PCR. Of the 34 periapical lesions studied, 59% showed HCMV and EBV dual infection, 27% showed only HCMV, 3% showed only EBV, and 12% showed neither HCMV nor EBV. Large size periapical lesions ($\geq 6 \text{ mm} \times 7 \text{ mm}$) and symptomatic lesions yielded significantly more herpes viruses than small lesions ($p < 0.001$). Fifty-five percent of asymptomatic lesions revealed neither HCMV nor EBV. The most common bacteria isolated were *Streptococcus*, *Fusobacterium*, *P. micros*, *Staphylococcus*, and *Campylobacter*. Black-pigmented anaerobic rods (*P. gingivalis*, *P. endodontalis*, *P. intermedia*, and *P. nigrescens*) were found exclusively in symptomatic lesions. The *Fusobacterium* species was the most common isolate in periapical lesions that contained HCMV plus EBV or were large in size. *Staphylococcus* species were common in periapical lesions that were negative for HCMV and EBV, asymptomatic, or small ($\leq 5 \text{ mm} \times 6 \text{ mm}$). The authors hypothesized that herpes viruses have several virulence factors that may contribute to periapical pathosis, including the ability to induce immune impairment and subsequent overgrowth of pathogenic microorganisms. The authors further concluded herpes viruses may also contribute to periapical pathosis by inducing cytokine and chemokine release from inflammatory and non-inflammatory host cells.

The presence of fungi has been observed in primary root canal infections and in cases refractory to endodontic therapy. Baumgartner et al. (98) used PCR to determine the occurrence of the fungus *C. albicans* in 24 samples taken from infected root canals and 19 aspirates of cellulitis or abscesses of endodontic origin. The presence of *C. albicans* was detected by using PCR with specific primers directed against the 18S rRNA gene segment of the organism. *Candida albicans* was detected in 21% of the samples

from infected root canals and none of the 19 aspirates of cellulitis or abscesses, indicating that PCR is a viable method for identification of *C. albicans* in infected root canals. Sen et al. (99) observed 10 maxillary and mandibular molars with periapical lesions under SEM and found yeasts heavily invading the root canals of 4 samples. Siqueira et al. (100) investigated the microbial colonization patterns in primary root canal infections and found yeast-like cells in conjunction with bacteria in 1 of 15 teeth examined by SEM.

Waltimo et al. (101) studied the occurrence of fungi in 967 endodontic samples taken from root canals associated with persistent periradicular infection. Fungi were found in 7% of the samples, either in pure culture (6 samples) or in conjunction with bacteria (41 samples). The most commonly isolated species was *C. albicans*. Other fungal species isolated were *C. glabrata*, *C. guilliermondii*, *C. inconspicua*, and *Geotrichium candidum*. Alpha- and non-hemolytic streptococci were the most common bacteria isolated in conjunction with the fungi. Sundqvist et al. (102) and Molander et al. (103) also recovered *C. albicans* from the root canals of teeth with failed endodontic treatment and chronic periradicular abscesses.

Treponemes have also been implicated in the pathogenesis of periradicular disease. Baumgartner et al. (104) collected samples from 54 asymptomatic infected root canals and aspirates from 84 cases of endodontic abscesses or cellulitis and used PCR to detect the presence of spirochetes. Spirochetes were detected in 61% of aspirates from abscesses or cellulitis and from 37% of samples from asymptomatic infected root canals. The most frequently encountered organism was *T. socranskii*, followed by *T. maltophilum*, *T. denticola*, *T. pectinovorum*, and *T. vincentii*.

Siqueira et al. (105) used 16s rDNA PCR to detect the occurrence of *T. denticola* in 54 infected root canals. The authors found *T. denticola* in 35% of asymptomatic cases, 53% of cases with sensitivity to percussion, and 50% of teeth with an acute periradicular abscess. The organism was found in a total of 43% of cases. The authors concluded that *T. denticola* may have a role in the pathogenesis of periradicular disease.

Siqueira et al. (106) also used 16s rDNA PCR to detect the occurrence of *T. socranskii* in 60 infected root canals. The authors found *T. socranskii* in 39% of asymptomatic cases, 42% of cases with acute apical periodontitis, and 25% of teeth with an acute periradicular abscess. The organism was found in a total of 35% of cases. The authors could not demonstrate a relationship between *T. denticola* and the occurrence of symptoms. The authors concluded that *T. socranskii* may have a role in the pathogenesis of different forms of periradicular disease.

These studies indicated the microbial flora of the necrotic root canal is complex and consists of a variety of microorganisms. Molecular methods are increasingly able to identify new inhabitants of the infected root canal and will likely elucidate new species as these techniques are further refined.

Bacteria Associated With Signs and Symptoms of Periradicular Disease

Many studies (107-116) associated black-pigmented bacteria with signs and symptoms of pulpal and periradicular disease. Sundqvist et al. (107) evaluated the prevalence of black-pigmented *Bacteroides* in the root canals of 72 single-rooted teeth with necrotic pulps and apical periodontitis. All 72 root canals contained bacteria. Twenty-two root canals contained black-pigmented *Bacteroides* species, with *Bacteroides intermedius* and *Bacteroides endodontalis* the most common isolates.

Bacteroides gingivalis, *Bacteroides loescheii*, and *Bacteroides denticola* were also isolated from these root canals. In total, 173 bacterial strains were isolated from these 22 root canals, 25 of which were strains of black-pigmented *Bacteroides* (30%). In root canals containing black-pigmented *Bacteroides*, 95% of the cultivable bacteria were anaerobic. Acute apical abscesses and purulent drainage through the root canal was associated with 16 of the 22 root canals containing black-pigmented *Bacteroides* species. The authors suggested that specific combinations of bacteria in the root canal may induce purulent inflammation in the apical region, and the presence of *B. endodontalis*, *B. gingivalis*, or *B. intermedius* in the bacterial population was required.

Van Winkelhoff et al. (108) examined the presence of black-pigmented *Bacteroides* in abscesses of endodontic and periodontal origin. Twenty-six of 28 odontogenic abscesses sampled contained one or more species of black-pigmented *Bacteroides*, with *Bacteroides endodontalis* isolated almost exclusively from isolates of endodontic origin.

Griffie et al. (109) studied the relationship of *Bacteroides melaninogenicus* to the symptoms associated with pulpal necrosis. Thirty-three cariously and traumatically exposed dental pulps were sampled and cultured anaerobically using pre-reduced thioglycollate broth and selective blood agar plates for *B. melaninogenicus*. The presence of *B. melaninogenicus* was significantly ($p < 0.05$) associated with pain, sinus tract formation, and foul odor. The authors also suggested a relationship existed between this microorganism and apical tissue sensitivity and localized swelling. The authors demonstrated growth in thioglycollate broth ranging from 88% at the initial appointment to 72% at the obturation appointment.

Heimdahl et al. (110) isolated a total of 174 anaerobic and 22 aerobic bacterial species from 58 patients with acute orofacial infections of odontogenic origin. Anaerobic Gram-negative rods were more frequently isolated from infections classified severe than those classified as mild. The authors stated that *Fusobacterium nucleatum* appeared to be related to the severity of the odontogenic infection.

Hashioka et al. (111) evaluated the relationship between symptoms and anaerobic bacteria in infected root canals. The bacterial flora from 28 teeth with apical periodontitis from 25 patients was cultured and correlated to the presence of symptoms. The authors found that *Eubacterium* was significantly ($p < 0.05$) associated with acute or chronic clinical symptoms, while *Peptostreptococcus*, *Peptococcus*, and *Porphyromonas gingivalis* was associated with subacute clinical symptoms. Percussion pain was significantly ($p < 0.05$) related to the presence of *Bacteroides*, and *Porphyromonas* and *Bacteroides* were significantly ($p < 0.05$) related to odor in the infected canals.

Gomes et al. (67,112) described the associations between specific symptoms and bacteria or combinations of bacteria in infected root canals. Seventy root canals were cultured and correlated to the presence of clinical signs and symptoms. The authors found significant associations between pain and the presence of *Prevotella* spp. or *Peptostreptococcus* spp. ($p < 0.01$), between percussion sensitivity and *Prevotella* spp. ($p < 0.01$), between swelling and *Eubacterium* spp ($p < 0.01$) or *Prevotella* spp. and *P. micros* ($p < 0.05$), and between purulent exudates and *F. necrophorum* ($p < 0.01$) or *P. loescheii*, *S. constellatus*, and *Bacteroides* spp ($p < 0.05$). Highly significant ($p < 0.001$) positive associations were found between *Peptostreptococcus* spp. and *Prevotella* spp., between *Peptostreptococcus* spp. and *P. melaninogenica*, between *P. micros* and

Prevotella spp., *P. micros* and *P. melaninogenica* and between *Prevotella* spp. and *Eubacterium* spp. The authors concluded that clinical signs and symptoms are related to a variety of species and combinations of species contained in the endodontic microflora.

Haapasalo et al. (113,114) analyzed the incidence of black-pigmented *Bacteroides* spp. in 35 acute and 27 clinically asymptomatic cases of apical periodontitis. The authors found *Bacteroides* spp. were always part of a polymicrobial infection, with *B. intermedius* the most frequently isolated species followed by *B. denticola*. Various species of *Bacteroides* were associated with the presence of symptoms. *Bacteroides gingivalis*, *B. endodontalis*, and *B. buccae* were usually found in cases of acute apical periodontitis, *B. intermedius* was found in cases of either acute or asymptomatic apical periodontitis, and *B. denticola* was mostly restricted to asymptomatic cases. *Mitsuokella dentalis*, a *Bacteroides*-like organism, increased the probability that acute symptoms would persist beyond 1 week after the beginning of treatment.

In contrast, Baumgartner et al. (115) failed to show a significant relationship between the presence of black-pigmented bacteria and the occurrence of clinical signs or symptoms associated with endodontic infections. Microbial samples from 40 root canals were collected and cultured using conventional laboratory methods and 16S rRNA PCR for differentiation of *P. nigrescens* from *P. intermedia*. Black-pigmented bacteria were found in 22 of 40 samples, with sixteen of these cases associated with purulent drainage.

Siqueira et al. (116) also failed to show a significant relationship between the presence of *Bacteroides forsythus* and the occurrence of clinical symptoms. Species-specific nested PCR was used to detect the presence of *B. forsythus*. The authors detected *B. forsythus* in 13 of 22 asymptomatic cases, 4 of 10 root canals associated with

acute apical periodontitis, and 9 of 18 cases diagnosed with acute periradicular abscesses. *Bacteroides forsythus* was found in 26 of 50 endodontic infections; however, the authors found no relationship between this organism and the occurrence of symptoms.

The Influence of Infection on the Success of Endodontic Therapy at the Time of Root Canal Obturation

Sjögren et al. (117) analyzed the influence of infection at the time of obturation on endodontic success and failure. Fifty-five single-rooted teeth with clinically diagnosed apical periodontitis were instrumented with a step-back technique using 2.5% sodium hypochlorite irrigation. Microbial samples were taken from the root canal before and following instrumentation and examined for the presence of viable bacteria. All initial samples were positive for bacteria, while 22 of 55 post-instrumentation samples demonstrated bacterial growth. Teeth that yielded a negative culture exhibited complete periapical healing after 5 years in 94% of the cases. Teeth with a positive culture showed complete healing in 68% of the cases. The authors concluded the persistence of bacteria negatively impacts periapical healing and suggested that complete elimination of the root canal flora cannot be achieved in a single appointment.

Sundqvist et al. (102) and Molander et al. (103) also suggested the persistence of bacteria in the root canal may have a negative impact on periapical healing. Each author demonstrated the presence of bacteria in root-filled teeth with periapical lesions. This microbial population found in the root canal following obturation was characterized as mono-infectious with equal proportions of facultative and obligate anaerobes, a flora distinctly different from that found in un-treated canals. Sundqvist et al. (102) demonstrated an average of only 1.3 bacterial species per canal with bacteria, with 42%

of the bacteria present anaerobic. Molander et al. (103) demonstrated an average of only 1.7 bacterial species per canal, with 39% of the bacteria present anaerobic.

Nair et al. (118) used light and electron microscopy to examine 9 therapy-resistant asymptomatic periapical lesions removed as block biopsies during surgical treatment of the involved teeth. The biopsies were taken from 4 to 10 years after non-surgical endodontic treatment. Microorganisms were present in the apical portion of the root canal in 6 of 9 biopsy specimens. Four of the lesions demonstrated bacteria, and two demonstrated yeast. The authors concluded the presence of microorganisms in the apical root canal may play a significant role in endodontic treatment failures.

Bacteria clearly have an important role in pulpal and periradicular pathosis. Empirically, these studies implied that control or elimination of the root canal infection would likely increase the success of endodontic therapy.

Bacteria have also been identified in dentinal tubules (119-121). Hoshino et al. (119) demonstrated bacterial invasion of 9 non-exposed dental pulps of freshly extracted teeth. The pulps were determined by examination and electric pulp tests to be clinically sound beneath intact dentin. Bacteria were present in 6 out of 9 non-exposed pulps and resembled the microbial population present in deep dentinal lesions, with the most common species including *Eubacterium* spp., *Propionobacterium* spp., and *Actinomyces* species. The authors concluded the close association between the bacteria cultured and the bacteria seen in deep dentinal lesions suggests that bacteria travel through dentinal tubules to reach the pulp.

Love et al. (120) studied bacterial penetration through dentinal tubules in different areas of human roots. Extracted, single-rooted teeth were instrumented, sectioned

longitudinally, and immersed in a suspension containing *S. gordonii* for 3 weeks. Heavy infection was found in the cervical and mid-root areas, with penetration into the dentinal tubules up to 200 μm . Mild infection was found in the apical area, with penetration extending 60 μm into the dentinal tubules.

Nagaoka et al. (121) analyzed the difference in bacterial penetration rates through dentinal tubules of vital and necrotic teeth. Pulpectomy and root canal filling were performed unilaterally on 19 intact maxillary third molars 30 or 150 days prior to extraction. The contralateral third molar was not instrumented or obturated. Class V cavity preparations were prepared bilaterally on the palatal surfaces of each third molar and left un-restored. At 150 days, the bacterial penetration rate was significantly higher in necrotic teeth. The authors concluded that vital pulps are more resistant to bacterial invasion than necrotic pulps.

Drake et al. (122) analyzed the effect of the smear layer on bacterial retention in 26 extracted human canines. Step-back instrumentation and irrigation with 2.5% sodium hypochlorite was used to prepare each canal. Following preparation, teeth were irrigated with either 20 mL of sterile saline or two 10 mL increments of 17% EDTA followed by 10 mL of 2.5% NaOCl and inoculated with a suspension containing *S. anginosus*. Teeth with the smear layer left intact harbored 10^4 colony-forming units of bacteria, and teeth with the smear layer removed harbored 10^5 colony-forming units. The authors concluded the smear layer produced during instrumentation of the root canal may inhibit bacterial colonization due to the blockage of dentinal tubules.

Perez et al. (123) evaluated the effect of the smear layer on bacterial invasion of dentinal tubules. Forty-eight bovine dentin samples were either instrumented leaving the

smear layer intact or left un-instrumented and treated with either 17% EDTA and 2.5% NaOCl. Each sample was inoculated with *S. sanguis* and placed in a receiver broth for evaluation of bacterial penetration through dentinal tubules. Samples with the smear layer removed demonstrated growth in the receiver broth, and teeth with the smear layer left intact revealed an absence of growth in 88% of cases. The authors concluded the smear layer limits bacterial penetration through dentinal tubules in vitro.

These studies indicated that bacteria are able to occupy unique ecological niches such as dentinal tubules and the smear layer. Further research is needed to evaluate the importance of viable bacteria in these areas of the root canal.

Bacterial Reduction During Instrumentation

Many authors (28-31,124-127,129-137) analyzed the effectiveness of various instrumentation regimens on reducing intracanal bacteria. Byström and Sundqvist (28) cultured 17 single-rooted necrotic teeth with periapical lesions after instrumentation with stainless steel files and irrigation with sterile saline. The authors found a reduction in bacterial numbers from 10^4 to 10^6 in the initial samples to 10^2 to 10^3 following instrumentation. No teeth cultured bacteria-free after the first appointment. Bacteria persisted in 7 root canals despite treatment on 5 successive occasions. Byström et al. (124-126) performed additional studies to determine the antibacterial efficacy of various steps in endodontic treatment. Necrotic teeth with periapical lesions were instrumented using stainless steel files in a filing or reaming motion and 0.5% NaOCl, 0.5% NaOCl and EDTA, camphorated paramonochlorophenol, camphorated phenol, or calcium hydroxide as irrigants or inter-appointment dressings. The authors were only able to achieve predictable negative cultures following instrumentation with NaOCl and EDTA

as irrigants and calcium hydroxide as an inter-appointment dressing. Teeth with negative cultures before obturation were found to have a 95% success rate (127), demonstrating the benefit of a bacteria-free canal at the time of root canal filling.

Dalton et al. (29) compared the ability of 0.04 tapered nickel-titanium rotary instruments and K-flex files in a step-back instrumentation sequence to reduce intracanal bacteria in 48 patients with apical periodontitis. Sterile saline was used for irrigation. Bacterial samples were taken before, during, and after instrumentation. The authors demonstrated complete elimination of bacteria in only 28% of samples with no significant differences between the two instrumentation regimens. The authors stated the high percentage of positive cultures in the molar samples may be due to communications between the mesiobuccal canal and the un-instrumented mesiolingual canal. This may have been the problem, since Kerekes and Tronstad (128) demonstrated that approximately 65% of mandibular molars have a direct communication. The authors concluded the addition of antimicrobial irrigants such as sodium hypochlorite to the instrumentation regimen is important in reducing intracanal bacteria.

Shuping et al. (30) evaluated the extent of bacterial reduction with nickel-titanium rotary instrumentation and 1.25% NaOCl irrigation. The additional antibacterial effect of placing calcium hydroxide for greater than 1 week was also tested. Forty-two subjects with radiographic and clinical signs of chronic apical periodontitis were recruited. The canals were sampled before treatment, during and after instrumentation, and after treatment with calcium hydroxide and the samples were incubated anaerobically for 7 days at 37°C. The bacteria from each sample were quantified and the log₁₀ values were used for calculations and comparisons. The initial sample confirmed infection of the

canals. A significantly greater pattern of reduction of bacteria was observed when NaOCl was used as an irrigant, compared with the results of the Dalton et al. (29) study, in which sterile saline was used as the irrigant ($p < 0.05$). After instrumentation with NaOCl irrigation, 61.9% of canals were rendered bacteria-free. The placement of calcium hydroxide for at least 1 week rendered 92.5% of the canals bacteria free. This was a significant reduction, compared with NaOCl irrigation alone ($p = 0.0001$). The results of this study indicated that NaOCl irrigation with rotary instrumentation is an important step in the reduction of canal bacteria during endodontic treatment. However, this method could not consistently render canals bacteria-free.

Ørstavik et al. (129) analyzed the effect of extensive apical reaming and calcium hydroxide therapy on reducing bacteria in 23 teeth with a radiographic diagnosis of apical periodontitis. Each tooth was treated in two appointments using extensive apical reaming without antimicrobial agents and with a one-week treatment of calcium hydroxide. Bacterial samples were taken initially and at the completion of each appointment. Thirteen of 14 samples were positive initially for bacterial growth. Fourteen of 23 samples were positive for bacterial growth following the first appointment, and 8 of 23 were positive following the second appointment. Only 1 of 8 positive samples showed higher numbers at the beginning of the second appointment following calcium hydroxide therapy. Growth was detected in the other 7 samples but was reported as non-quantifiable. The remaining fifteen teeth were negative for bacteria at the end of the second appointment. The authors also indicated the success of the calcium hydroxide may be attributed to the inability to effectively remove the entire medicament from the canal space prior to bacterial sampling.

Card et al. (31) also studied the effectiveness of increased apical enlargement in reducing intracanal bacteria in teeth with a clinical and radiographic diagnosis of apical periodontitis. Two mandibular canines, 11 premolars, and 2 molars were instrumented using 0.04 taper ProFile® rotary files and 1% sodium hypochlorite irrigation. The canines and premolars were instrumented to a #8 size and the molars to a #7 size. Final instrumentation was performed with LightSpeed® files and 1% NaOCl irrigation to a size #60 for molars and size #80 for canines and premolars. Bacterial samples were taken initially as well as after instrumentation with the ProFile® rotary files and LightSpeed® files. The bacteria from each sample were quantified and the log₁₀ values were used for calculations and comparisons. The initial sample confirmed infection of the canals. The authors demonstrated 100% bacterial reduction in the canine and premolar samples and 81.5% reduction in the molar samples after the first instrumentation. Following the second instrumentation, 89% of the molar samples were bacteria-free. In molar samples without a clinically detectable communication, 93% were bacteria-free after the first instrumentation. The authors concluded a high percentage of teeth may be rendered bacteria-free using this instrumentation regimen and may preclude the use of an antimicrobial dressing between appointments. The authors also stated that instrumentation to sizes used in this study may predispose a tooth to fracture, although the safe limits of apical instrumentation have not been determined.

Coldero et al. (130) compared in vitro intracanal bacterial reduction using nickel-titanium rotary instruments with and without apical enlargement. Thirty-eight palatal roots of maxillary molar teeth with mature apices were subdivided according to lengths and then randomly assigned to two experimental and one control group. The roots were

sterilized and then re-infected with *Enterococcus faecalis*, which served as a bacteriologic marker. All roots in the experimental groups were prepared in a step-down sequence with engine-driven ProFile® GT® rotary files at 350 rpm. In experimental group A (n = 16) additional apical enlargement to ISO size #35 was performed. In group B (n = 16) a serial step-back technique was followed with no apical enlargement. Instrumentation was combined in groups A and B with irrigation using 4.4% NaOCl and 15% EDTA. In the control group (group C, n = 6) only irrigation was carried out with no mechanical preparation. Samples were then taken from the root canals to determine the numbers of remaining bacteria. In groups A and B, 15 (94%) and 13 (81%) specimens were rendered bacteria-free, respectively. In the control group, none of the specimens were bacteria-free. A significant difference ($p < 0.001$) was observed in the antibacterial effects of the experimental and control regimens. However, no significant differences ($p = 0.276$) were found between the preparation methods used in the experimental groups. No significant differences were also found in intracanal bacterial reduction when ProFile® GT® rotary preparation with NaOCl and EDTA irrigation was used with or without apical enlargement preparation techniques. Therefore, removal of dentin in the apical part of the root canal may not be necessary when a suitable coronal taper is achieved to allow satisfactory irrigation of the root canal system with antimicrobial agents.

Rollison et al. (131) compared in vitro the antibacterial effectiveness of two nickel-titanium rotary instrumentation systems differing in sequence and apical enlargement size. Forty-four mesiobuccal canals of mandibular molars inoculated with radioactively labeled *E. faecalis* were instrumented either to an apical size #35 with GT®

and ProFile[®] rotary files or to an apical size #50 with Pow-R[®] rotary instruments. Phosphate-buffered saline was used for irrigation. The authors found that instrumentation to an apical size #50 with Pow-R[®] rotary instruments significantly reduced more intracanal bacteria than GT[®] or ProFile[®] rotary files ($p < 0.05$).

Siqueira et al. (132) evaluated in vitro the ability of hand Nitiflex files, GT[®] files, and ProFile[®] 0.06 Tapers[™] Series 29[™] to reduce intracanal populations of *E. faecalis*. Sterile saline was used for irrigation. Each instrumentation regimen significantly reduced the number of viable bacteria in the root canal. Instrumentation to a Nitiflex #40 file reduced intracanal bacteria significantly greater than any other technique tested. The authors concluded these instrumentation techniques along with sterile saline irrigation could effectively eliminate greater than 90% of intracanal bacteria.

Siqueira et al. (133) evaluated the in vitro intracanal bacterial reduction produced by instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite or saline solution. Root canals inoculated with *Enterococcus faecalis* were instrumented to an apical size #40 Nitiflex file using an alternating rotary motion technique (described below) and irrigated with the solutions tested. Canals were sampled before and after preparation. After serial dilution, samples were plated onto Mitis salivarius agar, and the colony-forming units were counted. Inhibitory effects of the three NaOCl solutions on *E. faecalis* were also evaluated by means of the agar diffusion test. All test solutions significantly reduced the number of bacterial cells in the root canal ($p < 0.05$). No significant differences were found between the three NaOCl solutions tested ($p > 0.05$). Nonetheless, all NaOCl solutions were significantly more effective than saline solution in reducing the number of bacterial cells within the root canal ($p < 0.05$). The three NaOCl

concentrations showed large zones of inhibition against *E. faecalis*. The results of this study suggested that regular exchange and the use of large amounts of irrigant should maintain the antibacterial effectiveness of the NaOCl solution, compensating for the effects of concentration.

Siqueira et al. (134) compared in vitro the ability of the alternated rotary motions (ARM) technique; hand nickel-titanium files and irrigation with 2.5% NaOCl; ARM technique and combined irrigation with 2.5% NaOCl and citric acid; ARM technique and combined irrigation with 2.5% NaOCl and 2% chlorhexidine gluconate; and GT[®] rotary files with 2.5% NaOCl irrigation to reduce intracanal populations of *E. faecalis*. A control group consisted of 10 root canals irrigated with 0.85% sterile saline solution. The ARM technique was completed according to the following regimen. A #25 Nitiflex file was inserted into the canal to a point where it bound slightly and then turned clockwise no more than one-quarter revolution. The file was then turned counterclockwise no more than one-quarter revolution with light apical pressure. These motions were continued until the file reached working length. Apical preparation was continued through a #40 Nitiflex file. The authors determined that each instrumentation and irrigation regimen significantly reduced the number of viable intracanal bacteria ($p < 0.05$). The mean reduction in bacterial numbers ranged from 63% for canals irrigated alternately with NaOCl and chlorhexidine gluconate to 78% for canals irrigated alternately with NaOCl and citric acid. No significant differences were found between each instrumentation and irrigation regimen. The authors concluded the use of antimicrobial irrigants is necessary to effectively eliminate bacteria regardless of the instrumentation technique utilized.

Pataky et al. (135) evaluated the ability of a step-back preparation technique with K-files or K-Nitiflex files, a standardized technique with K-reamers, and irrigation with sterile physiologic saline to reduce intracanal populations of *E. faecalis* in 40 teeth extracted for orthodontic purposes. Teeth in the step-back technique group were instrumented to a #25 K-file or Nitiflex master apical file. The largest master apical files in either the step-back or standardized instrumentation group was a size #40. Each tooth was irrigated using 0.1 mL of sterile saline after each instrument. The authors observed significant ($p < 0.01$) reductions in bacterial numbers with each mechanical instrumentation group compared to a no treatment control. No significant differences were found between each instrumentation group ($p > 0.05$), and no canals were rendered bacteria-free.

Peters et al. (136) analyzed the effect of instrumentation, irrigation with 2% sodium hypochlorite, and calcium hydroxide treatment in reducing intracanal bacteria in 42 necrotic teeth with periapical radiolucencies. All teeth were asymptomatic and included 15 incisors, 6 canines, 8 single-canal premolars, and 13 single-canal distal roots of mandibular molars. The teeth were instrumented using Flexofiles in a modified double-flare technique to a master apical size file of at least #35 and either packed with a slurry of calcium hydroxide and saline or obturated with gutta-percha and AH-26 sealer. Two mL of 2% sodium hypochlorite were used for irrigation after each file. A final rinse with 5 mL of 2% NaOCl was used following the completion of instrumentation. Calcium hydroxide was left in the canal for a period of 4 weeks. Bacterial samples were taken prior to and after instrumentation and at the beginning and end of the second appointment for those teeth initially packed with calcium hydroxide. Microorganisms were found in

all initial bacterial samples. Anaerobes comprised 97% of the total bacterial population in the initial samples. The authors found the total number of colony-forming units of bacteria dropped significantly ($p < 0.05$) from 1.0×10^6 to 1.8×10^3 after instrumentation. Thirty-two specimens (77%) had no cultivable bacteria after completion of initial instrumentation. The number of bacteria present in the canal at the start of the second visit for those teeth that received calcium hydroxide was significantly higher ($p < 0.05$) than the counts present after completion of initial instrumentation. The authors concluded that a calcium hydroxide slurry limits but does not prevent re-growth of intracanal bacteria, possibly due to the lack of direct contact between the calcium hydroxide and canal microorganisms in a clinical situation.

Piccolomini et al. (137) evaluated the efficacy of the Pumped Diodium-Nd:YAG laser in sterilizing contaminated root canals. After hand instrumentation, 30 teeth were inoculated with *Actinomyces naeslundii* CH-12 and 30 teeth with *Pseudomonas aeruginosa* ATCC 27853 and incubated for 24 hours. The teeth were divided into three subgroups: subgroup A received no treatment; subgroup B was irradiated with a laser (5 Hz for 15 seconds or 10 Hz for 15 seconds); and subgroup C was irrigated with 5.25% NaOCl. The number of viable bacteria in each group was evaluated by using the surface-spread plate technique. The results indicated an average 34% decrease in colony-forming units for *A. naeslundii* CH-12 and 15.7% for *P. aeruginosa* ATCC 27853 with the 5 Hz/15 second laser treatment and a 77.4% decrease for *A. naeslundii* CH-12 and 85.8% for *P. aeruginosa* ATCC 27853 with the 10 Hz laser frequency. No bacteria were detected in the canals treated with 5.25% NaOCl. The antibacterial effect of the Pumped

Diodium Nd:YAG laser was dependent on the radiation frequency. However, 5.25% NaOCl was more effective than either laser application.

These studies showed that conventional hand and rotary instrumentation techniques, irrigation with sodium hypochlorite, calcium hydroxide therapy, and laser application significantly reduces the number of viable intracanal bacteria. No current technique can predictably and repeatedly render canals bacteria-free.

Bacterial Reduction Following Ultrasonic Instrumentation and Irrigation

Huque et al. (59) evaluated ultrasonic intracanal irrigation procedures in eradicating bacteria from surface, shallow, and deep layers of root dentin using extracted human teeth. Rubbing a mixture of dental plaque and artificially decalcified dentin or carious dentin on root canal walls successfully produced artificial bacterial smear layer. Reservoir holes (3.5 mm in depth, 1 mm in diameter) were prepared parallel to the root canals on the de-crowned planes, and five separate bacterial species were placed (*Actinomyces israelii*, *Fusobacterium nucleatum*, *Propionobacterium acnes*, *Streptococcus mutans* and *Streptococcus sanguis*) in them. The Solfy ultrasonic machine at a power setting of 2 and size #15 endodontic files were placed in the root canal chamber and activated for 20 seconds during ultrasonic irrigation. Bacterial eradication after irrigation of the prepared canals was determined by bacterial recovery from the root canal surfaces and shallow layers where bacteria were smeared artificially and from deeper layers of root canal dentin reservoir holes. Ultrasonic irrigation with 5.5% and 12% NaOCl eradicated bacteria from artificial smear layer ($p < 0.0001$), while 12% NaOCl irrigation with a syringe was insufficient. Ultrasonic irrigation with water or 15% EDTA failed to eradicate bacteria from smeared surfaces. Ultrasonic irrigation with 12%

NaOCl killed *A. israelii*, *F. nucleatum*, *P. acnes*, *S. mutans*, and *S. sanguis* placed in reservoir channels, although for *F. nucleatum*, a very small number of bacteria remained in 5 samples out of 12. Ultrasonic irrigation with less concentrated NaOCl failed to eliminate bacteria completely from reservoir channels in most samples. Ultrasonic irrigation with 12% NaOCl appeared to eliminate bacteria efficiently from surface, shallow, and deep layers of root dentin.

In a study evaluating the antibacterial effect of ultrasonic instrumentation, Martin (32) used 5.5% sodium hypochlorite, 1% acid pentanediol, or a neutral buffer solution in conjunction with ultrasound. Extracted human molars were instrumented, sterilized, apically sealed, and inoculated with one of four test organisms. Ultrasound reduced the number of bacteria when used alone, but the method became significantly more bactericidal when it was combined with sodium hypochlorite or acid pentanediol.

Barnett et al. (138) evaluated the bacterial status of root canals following sonic (using the Endostar 5), ultrasonic (using the Cavi-Endo[®]), and hand instrumentation. Following induction of root canal infection in 50 single-rooted teeth in young dogs, each instrumentation technique was used with either saline or 2.5% sodium hypochlorite irrigant. After completion of instrumentation, a sterile paper point was sealed in each canal. Seven days later the root canals were opened and cultured. The results showed the sonic and ultrasonic techniques were no more effective in eliminating bacteria from the root canal than hand instrumentation. Irrigation with sodium hypochlorite and the Cavi-Endo[®] appeared to be more effective, antibacterially, than saline, but the results were not statistically significant.

DeNunzio et al. (139) studied the effectiveness of hand and Endosonic instrumentation to remove a standard inoculum of bacteria from the root canal systems in the premolars of dogs. A Cavi-Endo[®] unit was used as an ultrasonic source, and a step-back method used for hand instrumentation. Sterile saline was used as an irrigating solution in both groups. Teeth were inoculated and instrumented in vivo and the teeth were subsequently extracted. The authors found no significant differences in the removal of bacteria between the two methods of instrumentation. The authors concluded that selection of one of these two methods for canal instrumentation should be based on criteria other than the ability to remove bacteria from the root canal.

Ahmad (140) evaluated the effect of ultrasonic instrumentation, using the Enac dental unit, on the disruption of *Bacteroides intermedius*. Using the same detection method as was used in a previous study (45), the author determined that cavitation was not produced when the Enac unit was used with a #15 K-type file at a power setting of 1-3. Acoustic streaming was produced at these settings. Active bacterial cultures in test tubes were sonicated for 1, 5, or 15 minutes. The author found that an ultrasound exposure time of 1 minute killed 21.6% of bacteria, 5 minutes killed 30.4% of bacteria, and 15 minutes killed 92.9% of bacteria. Since bacteria remained vital, the author concluded that ultrasound has little practical importance in the disruption of bacteria.

Siqueira et al. (60) analyzed the effect of irrigation with 2 mL of 4.0% NaOCl following agitation with hand files, irrigation with 2 mL of 4.0% NaOCl following ultrasonic agitation, and irrigation with NaOCl alternated with hydrogen peroxide on the in vitro disruption of *E. faecalis*. Canals contaminated with *E. faecalis* and irrigated with sterile saline served as a control. Induction of turbidity in brain-heart infusion broth

tubes was used to identify the presence of remaining bacteria in the root canal. The authors found no significant differences between the three irrigation regimens. Each experimental group significantly eliminated more bacteria than the saline control group, again suggesting the importance of sodium hypochlorite in the disruption of root canal bacteria.

Fegan and Steiman (141) compared the in vitro ability of the Nd:YAG laser with saline and 5.25% NaOCl irrigation to hand and ultrasonic instrumentation with saline and 5.25% NaOCl irrigation in eliminating *Bacillus stearothersophilus* from single-rooted teeth. All instrumentation times were limited to 1 minute. The number of colony forming units was determined following inoculation onto blood agar plates. The authors determined that each group using sodium hypochlorite irrigation was effective in reducing the numbers of *B. stearothersophilus* from the root canal. Hand and ultrasonic instrumentation with sterile saline irrigation did not effectively remove bacteria from the root canal. The use of Nd:YAG laser irradiation was effective in reducing viable intracanal bacteria.

Weber et al. (142) evaluated the effect of passive ultrasonic activation of 2% chlorhexidine or 5.25% NaOCl irrigant on residual antimicrobial activity in 94 single-rooted extracted teeth. Each tooth was instrumented using a crown-down technique and irrigated with 2% chlorhexidine, 2% chlorhexidine plus one-minute of passive ultrasonic activation, 5.25% NaOCl, or 5.25% NaOCl plus one-minute of passive ultrasonic activation. Ten teeth irrigated with sterile phosphate buffered saline (PBS) served as negative controls. Ultrasonic activation was performed with the MiniEndo™ system with Zipperer size #20 files. Canals were further enlarged with a Parapost System drill to

provide a solution reservoir. Each canal was rinsed with PBS and stored for 6 hours. Six hours after instrumentation, 20 μ L of fluid were extracted from each canal and placed into agar wells containing *S. sanguis*. Sampling was performed at 24, 48, 72, 96, 120, 144, and 168 hours, and zones of inhibition were measured. The authors determined that residual antimicrobial activity with 2% chlorhexidine was significantly greater than 5.25% NaOCl irrigation alone and with passive ultrasonic activation. Chlorhexidine exhibited residual antimicrobial activity as long as 168 hours after instrumentation, while NaOCl showed little activity after 48 hours.

Spoleti et al. (57) evaluated in vitro the effect of passive ultrasonic activation on root canal disinfection. Sixty human teeth were used consisting of maxillary incisors, maxillary canines, and the distobuccal root of maxillary molars. An inoculum consisting of *S. aureus*, *S. viridans*, and *E. coli* was placed in each canal. Each tooth was then instrumented using a crown-down technique to an apical size #50 for the incisors and canines and #35 for molars. Passive ultrasonic activation was performed with 0.5 mL of sterile saline and a #20 K-file attached to a Cavi-Endo[®] for 10 seconds. Samples were obtained and plated on blood agar to identify and quantify remaining bacteria. Surviving colonies were found in each sample. The number of surviving colonies was higher when ultrasonics was not used. The authors concluded that ultrasonic activation is beneficial in disinfection of the root canal system, but it is difficult to use in roots of small sizes and complete disinfection cannot be achieved without antiseptic irrigants.

Sjögren and Sundqvist (55) analyzed the antibacterial effect of ultrasonic root canal instrumentation in 31 human, single-rooted necrotic teeth with periapical radiolucencies. Instrumentation was performed with a Cavi-Endo[®] using 0.5% NaOCl as

an irrigant. Three appointments were used for each subject, and no antibacterial dressing was used between each appointment. Bacterial samples were taken prior to and after instrumentation, after ultrasonic instrumentation, and at each successive appointment. All 31 teeth had bacteria present initially. The median number of cells before treatment was 3.5×10^4 . The authors determined that ultrasonic activation eliminated bacteria from canals more effectively than hand instrumentation alone, and concluded the use of an antibacterial dressing is necessary to achieve as complete a reduction in bacterial numbers as possible.

These studies showed that ultrasonic instrumentation and ultrasonic activation of antimicrobial irrigants is more effective in reducing intracanal bacteria than hand or rotary instrumentation alone, although neither method of instrumentation is capable of complete disinfection of the root canal system.

***E. FAECALIS* AND ITS ROLE IN PRE- AND POST-TREATMENT DISEASE**

Enterococcus faecalis has received considerable attention in the medical and dental literature as a significant factor in various forms of disease. Ruoff et al. (143) estimated that *E. faecalis* is responsible for approximately 80% of all infections caused by enterococci. Known for its ability to tolerate unique and challenging ecological conditions, *E. faecalis* was implicated in the development of nosocomial bacteremia and urinary tract infections, intra-abdominal infections, enterococcal endocarditis, and apical periodontitis following endodontic therapy (144-147).

Members of the genus *Enterococcus*, including *E. faecalis*, are Gram-positive, facultatively anaerobic, catalase-negative cocci. Enterococcal cells occur in pairs or short

chains in liquid media, do not form endospores, and are usually non-motile. Creamy whitish colonies and β -hemolysis are normally observed on blood agar. *Enterococcus faecalis* belongs to Lancefield serological group D and will exhibit growth at 10°C and 45°C, in 6.5% NaCl, at pH 9.6, and with 40% bile, which is a useful characteristic for identification (148). The habitat of *E. faecalis* includes the oral cavity, gastrointestinal tract, water, and food. Virulence factors contributing to the pathogenicity of *E. faecalis* include aggregation substance which facilitates attachment to cells (149), the surface protein *esp* which facilitates hydrophobic interactions with other cells (150), gelatinase which hydrolyzes extracellular proteins (151), hemolysin which lyses erythrocytes, neutrophils, and macrophages (152), and extracellular superoxide (153).

Several studies (154-159) have investigated the presence of *E. faecalis* in the root canals of teeth with an initial diagnosis of apical periodontitis. The results of these studies were mixed in terms of the presence or absence of *Enterococcus* spp. within the infected, necrotic root canal or periapical aspirates. Oguntebi et al. (154) performed permucosal aspiration of 10 periapical abscesses associated with necrotic teeth from healthy patients free of periodontal disease. Following disinfection of the oral mucosa with iodine tincture, a sterile 18-gauge needle was passed through the overlying mucosa and bone into the abscess and the contents withdrawn. The samples were incubated anaerobically and plated on selective agar for bacterial identification. A total of 25 bacterial strains were identified, with *F. nucleatum* and *S. mitis* the most frequently encountered isolates. No species of *Enterococcus* was isolated. De Sousa et al. (155) also failed to isolate species of *Enterococcus* from the root canals of 30 necrotic teeth with associated periapical abscesses. The most frequently isolated obligate anaerobes

were *P. prevotii*, *P. micros*, and *F. necrophorum*. Facultative anaerobes such as *G. morbillorum* and *S. mitis* were also found, but less frequently.

In contrast, Engström (156) isolated enterococci from 12% of culture-positive teeth at the onset of treatment of necrotic root canals. Siqueira et al. (157) also isolated *E. faecalis* from the root canals of 53 single-rooted necrotic teeth prior to treatment, 26 of which were asymptomatic and 27 of which were diagnosed clinically as acute periradicular abscesses. In this study, samples were analyzed for the presence of 13 bacterial species using whole genomic DNA probes and checkerboard DNA-DNA hybridization. The checkerboard DNA-DNA hybridization assay identified *E. faecalis* in 7.5% of total samples and 11.5% of asymptomatic samples.

Mejare (158) studied the occurrence of enterococci at the time of obturation on 612 root canals with a diagnosis of primary apical periodontitis. Ninety-two positive cultures were obtained, 30% of which contained enterococci. Specifically, the enterococci located were *S. faecalis* subsp. *faecalis*, *S. faecalis* subsp. *liquefaciens*, *S. faecalis* subsp. *zymogenes*, and atypical variants of *S. faecalis*, *S. faecium* var. *faecium*, and *S. faecium* var. *durans*. The author concluded enterococci may have a prominent impact on the prognosis of endodontic therapy.

Sirén et al. (159) compared the occurrence of *E. faecalis* in 40 *Enterococcus*-positive teeth and 40 *Enterococcus*-negative teeth where initial treatment did not result in healing. The results indicated that *E. faecalis* was isolated more frequently from teeth where the canal was unsealed and when treatment was performed over many appointments, indicating that compromised asepsis during endodontic therapy is a causative factor for *E. faecalis* contamination of the root canal.

E. faecalis is well recognized as a prominent bacterium in teeth with post-treatment apical periodontitis. Sundqvist et al. (102) determined the microbial flora present in 54 root-filled teeth with persistent periapical lesions and the outcome of conservative non-surgical re-treatment. Microbial samples were taken from the root canal after removal of the root filling without the use of solvents, at the second visit 7 days later (prior to hand instrumentation and irrigation with 0.5% NaOCl), and at the 3rd appointment following removal of the calcium hydroxide placed at the second visit. Nine of 24 canals positive for bacteria harbored *E. faecalis* as the only microorganism present in the canal. The overall success rate for conservative endodontic re-treatment was 73%. The success rate for teeth from which *E. faecalis* was isolated after removal of the prior root canal filling was 66%. The authors concluded the presence of infection at the time of obturation significantly decreases the success rate of teeth undergoing re-treatment.

Molander et al. (103) studied the microbial status of 100 root-filled teeth with radiographically verified apical periodontitis. Cultures were taken from 80 teeth with periapical pathosis and 20 teeth requiring re-treatment for technical reasons. Bacteria were found in 68% of teeth, with *E. faecalis* the most frequent isolate, found in 47% of culture-positive teeth. Enterococci were the most frequently isolated genera, demonstrating “heavy” or “very heavy” growth in 78% of culture positive teeth. Bacterial growth was sparse in teeth lacking periapical pathosis. The authors concluded the microflora of previously root-filled teeth differs from un-treated necrotic dental pulps.

Pinheiro et al. (160) studied the microbial flora present in 60 root-filled teeth with persistent periapical pathosis. Microbial samples were taken using paper points following removal of the prior root filling with Gates-Glidden drills and files without the

aid of chemical solvents. The samples were plated on selective agar and identified using advanced anaerobic culturing techniques. The results indicated that 85% of teeth were positive for bacteria, with *E. faecalis* the most frequently encountered isolate, present in 45% of all teeth and 53% of the culture-positive teeth.

The relative refractoriness of *E. faecalis* to inter-appointment dressings and root canal irrigants is of clinical concern. *E. faecalis* has demonstrated resistance to calcium hydroxide, camphorated paramonochlorophenol, camphorated phenol, antibiotic-steroid combinations, sodium hypochlorite, chlorhexidine digluconate, chlorhexidine acetate, and iodine compounds. Byström et al. (126) instrumented necrotic teeth with periapical radiolucencies using stainless steel files and 0.5% NaOCl, 0.5% NaOCl and EDTA, camphorated paramonochlorophenol, camphorated phenol, or calcium hydroxide as irrigants or inter-appointment dressings. Predictable negative cultures were only achieved following instrumentation with NaOCl and EDTA as irrigants and calcium hydroxide as an inter-appointment dressing. Calcium hydroxide alone proved to be ineffective against in vivo isolates of *E. faecalis*. Waltimo et al. (161) and Ørstavik and Haapasalo (162) also demonstrated the inability of calcium hydroxide to eliminate cultures of *E. faecalis* from experimentally infected dentinal tubules.

Gomes et al. (163) studied the in vitro ability of several concentrations of sodium hypochlorite and chlorhexidine gluconate to eliminate *E. faecalis*. Suspensions of pure cultures of *E. faecalis* in 0.85% saline were mixed ultrasonically for 10 seconds with each of the test irrigants, and placed in contact with the irrigants from 10 seconds to 30 minutes. Positive growth was determined by medium turbidity in brain heart infusion (BHI) broth. The results indicated that a thirty-second incubation time was required for

0.2% chlorhexidine gluconate liquid to eliminate *E. faecalis*, and a thirty-minute incubation time was required for 0.5% NaOCl to eliminate *E. faecalis*.

Evanov et al. (164) studied the ability of a calcium hydroxide solution and chlorhexidine to eliminate *E. faecalis* from dentinal tubules and to determine if the antimicrobial effect of these solutions is enhanced by heat. Ninety-five bovine mandibular central and lateral incisors were used in this study. Each tooth was sectioned into dentin disks 5 mm in height with the pulp lumens standardized to 2.5 mm in diameter. The smear layer on each disk was removed and each disk was sterilized in a steam autoclave. The lumens of each specimen were inoculated with 1×10^7 CFUs of *E. faecalis* in BHI broth and incubated for 72 hours. The BHI broth was then removed and chlorhexidine (0.12%), a 10% calcium hydroxide solution, or saline was introduced into the lumen and incubated at either 37°C or 46°C for 35 minutes. The specimen was then frozen, pulverized, and vortexed with 2 mL of PBS. Serial dilutions were made of each specimen, the samples were plated on BHI agar, and colony-forming units were counted 24 hours later. Chlorhexidine and the calcium hydroxide solution produced significantly less growth than saline. No significant difference was found between chlorhexidine and calcium hydroxide at the same temperature, and chlorhexidine or calcium hydroxide at 46°C produced significantly less growth than either group at 37°C ($p < 0.05$). The authors stated a 10% calcium hydroxide solution was used in this study because it could be manipulated into a thin mix that may penetrate dentinal tubules and reach the intended bacteria. The irrigant was left in the lumen of the disk for 35 minutes to simulate the amount of time clinically the irrigant would be in contact with the tooth surface. The authors concluded that delivering 10% calcium hydroxide or 0.12% chlorhexidine at

46°C increases the antimicrobial activity of each solution without the addition of more toxic materials such as CMCP.

Schäfer and Bössmann (165) studied the effectiveness of 2% chlorhexidine and calcium hydroxide, separately and combined, as intracanal dressings in extracted single-rooted human teeth with a straight canal. Each canal was instrumented to a size #40 file and the smear layer was removed. An inoculum of *E. faecalis* at a concentration $\geq 10^9$ CFU/mL was injected into each tooth and the sample was incubated aerobically. The specimens were then filled with calcium hydroxide paste, 2% chlorhexidine solution, or calcium hydroxide plus chlorhexidine in equal proportions and then incubated for 3 days. Dentin samples were taken from each canal using sterile Hedström #45-60 files after the canals were rinsed with sterile saline, transferred to culture media with inactivating substances, and colony-forming units were counted after a three-day incubation period. The results showed the chlorhexidine group was significantly more effective than the calcium hydroxide group ($p < 0.01$) or the calcium hydroxide plus chlorhexidine group ($p < 0.05$). No specimens were free of bacteria in the calcium hydroxide group or the calcium hydroxide plus chlorhexidine group after enlargement to a size #60 file. This study further demonstrated the ineffectiveness of calcium hydroxide in removing *E. faecalis* from infected root canals.

Cwikla et al. (166) studied the efficacy of calcium hydroxide, calcium hydroxide mixed with iodine-potassium iodide (IKI), and calcium hydroxide mixed with iodoform and silicone oil in disinfecting dentinal tubules contaminated with *E. faecalis*. Forty-five extracted human maxillary anterior teeth were enlarged to a standard diameter of 1.6 mm and the smear layer was removed. The specimens were autoclaved and then incubated in

the presence of 1.5×10^8 CFU/mL of *E. faecalis* for 21 days. Following incubation, the medications listed above were placed into the lumen of each specimen and the specimens were incubated for 7 days. After removing the medications, sterile carbide round burs with diameters of 1.8 mm and 2.1 mm were used to sequentially remove dentin powder from the inner surface of the tooth. The dentin powder was collected into tubes containing trypticase soy broth (TSB) media and the number of colony-forming units was determined following an overnight incubation of the media onto blood agar plates. The results showed that calcium hydroxide mixed with iodoform and silicone oil was significantly more effective in removing *E. faecalis* from the dentinal tubules than the other treatment groups ($p < 0.05$). The results also showed that significantly more bacteria were recovered in deeper dentin samples after treatment with calcium hydroxide alone. *E. faecalis* was shown to penetrate dentinal tubules up to 300 μm . The authors concluded the superior effects of calcium hydroxide mixed with iodoform and silicone oil may be due to the oily vehicle, which prolongs the action of the medicament.

Peciuliene et al. (167) studied the occurrence of yeasts and enterococci in 40 symptom-free and root-filled teeth with chronic apical periodontitis. The teeth were divided into 2 treatment groups. Group A received an inter-appointment dressing of calcium hydroxide for 10 to 14 days prior to obturation, and group B received a five-minute irrigation with iodine potassium iodide (IKI) followed by permanent filling of the root canal. Microbial samples were taken before and after chemomechanical preparation with reamers and Hedström files using 2.5% NaOCl and 17% EDTA irrigation and after iodine irrigation for samples in group B. *E. faecalis* was isolated initially from 21 of 33 culture positive teeth and in 6 of 10 teeth positive for growth following chemomechanical

preparation. All samples except one were free of bacterial growth following irrigation with IKI. The authors concluded that IKI may be a useful adjunct in the elimination of *E. faecalis* from the root canal system.

Evans et al. (168) investigated the mechanisms involved in the resistance of *E. faecalis* to the high pH of calcium hydroxide. *E. faecalis* strain JH2-2 was challenged by exposure to 0.005% NaOCl or calcium hydroxide solutions at a pH of 11.1 or 11.5. Cells were removed at 0, 15, and 30 minutes and viable bacterial counts were determined by serial dilution and plating onto BHI agar. The number of cells present initially was 2.3×10^9 per 10 mL. Exposure of the cells to 0.005% NaOCl resulted in $1.2 \times 10^{-4}\%$ of cells surviving after 15 minutes and $4.4 \times 10^{-6}\%$ cell survival after 30 minutes. Pre-treatment of the initial culture with 0.0001% NaOCl or calcium hydroxide at a pH of 10.3 for 30 minutes did not induce tolerance or enhance survival of *E. faecalis* in 0.005% NaOCl. Exposure of the cells to calcium hydroxide at a pH of 11.1 for 30 minutes resulted in 0.4% of the cells surviving and calcium hydroxide at a pH of 11.5 resulted in $1.3 \times 10^{-6}\%$ of the cells surviving. Pre-treatment with either 0.001% NaOCl or calcium hydroxide at a pH of 10.3 for 30 minutes did not induce tolerance or enhance survival of *E. faecalis* to further exposure of calcium hydroxide. The effect of blocking protein synthesis on *E. faecalis* cell survival was determined by pre-treating the cells with chloramphenicol. No discernible difference in cell survival was determined in the presence of chloramphenicol, suggesting that stress-induced protein production is not important for survival of *E. faecalis* at a high pH. The effect of blocking proton pumps was determined by pre-treating the cells with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a proton pump inhibitor. Cells exposed to calcium hydroxide at a pH of 11.1 for 30 minutes

displayed a 20-fold reduction in cell survival in the presence of CCCP. These results showed that a functioning proton pump, responsible for pumping cations into the cell to lower the internal pH, is critical for the survival of *E. faecalis* in a highly alkaline environment.

Figdor et al. (169) studied the ability of *E. faecalis* to endure extended periods of starvation in vitro. *E. faecalis* strain JH2-2 was grown and placed in various nutrient-limited media, including phosphate- and glucose-limited chemically defined media (CDM), amino acid-free CDM, PBS, and water. Starvation survival was determined by viable counts of serial dilution in PBS and plating on CDM agar. In addition, the recovery of starved cells by placement in pooled human serum (PHS) was also determined. The results indicated an initial rapid fall in cell numbers; however, a small remaining population of *E. faecalis* was able to survive for 4 months in water and for greater than 4 months in nutrient-limited media. The initial cell density was important for survival. Cell densities greater than 10^8 CFU/mL at the onset of starvation permitted *E. faecalis* long periods of survival. Starved cells recovered in 50% PHS were capable of growing and surviving for more than 4 months. The authors concluded that *E. faecalis* is capable of enduring long periods of starvation under conditions that it is likely to experience in many of its natural environments.

Shabahang and Torabinejad (170) studied the effect of a mixture of a tetracycline isomer, citric acid, and the detergent Tween 80 (BioPure™ MTAD™) on *E. faecalis*-infected root canals of 85 extracted single-rooted human anterior teeth. Each tooth was placed in a suspension of *E. faecalis* in BHI broth at a concentration of 1×10^8 cells/mL for 4 weeks in order to allow penetration of the bacteria into the dentinal tubules. Each

canal was instrumented using a combination of the passive step-back technique and rotary instruments to an apical ISO size #35 with 1 mL of 1.3% or 5.25% NaOCl irrigant used after each instrument size. The samples were divided into 5 treatment groups following cleaning and shaping. Group A received a five-minute exposure to MTAD™, group B a five-minute exposure to 1.3% NaOCl, group C a five-minute exposure to 5.25% NaOCl, group D a one-minute exposure to 17% EDTA and five-minute exposure to 1.3% NaOCl, and group E a one-minute exposure to 17% EDTA and five-minute exposure to 5.25% NaOCl. Growth of bacteria was determined by the presence of turbidity in BHI broth after incubation for 1 week. In addition, a sterile #4 round bur was used to prepare dentin shavings from 2 sites initiating on the external surface of the root and moving inward toward the root canal. The shavings were collected on BHI plates and incubated for 48 hours to determine the presence of viable bacteria within the dentinal tubules. No growth was observed in teeth in group A following incubation. Six of 15 teeth were positive in group B, 7 of 15 were positive in group C, 6 of 15 were positive in group D, and 5 of 15 were positive in group E. No additional growth was determined from the dentin shavings in group A. Two additional samples were positive from dentin shavings in group B, 1 additional sample was positive in group C and group D, and 3 additional samples were positive in group E. Fisher's exact test showed the combination of 1.3% NaOCl and a five-minute final rinse with MTAD™ was significantly more effective against *E. faecalis* than any other regimen ($p < 0.05$). This study further demonstrated the ineffectiveness of NaOCl to consistently disinfect root canals. Both 1.3% and 5.25% NaOCl left nearly 50% of root canals contaminated with *E. faecalis*. The authors stated the effective antimicrobial action of the doxycycline in

MTAD™ may be due to its low pH, its anti-collagenase activity, and its ability to bind to dentin and be slowly released over time.

These studies demonstrated the prominent role of *E. faecalis* in post-treatment endodontic disease, the ability of this bacterium to colonize well within dentinal tubules and avoid contact with intracanal medicaments, and the relative ineffectiveness of endodontic irrigants and inter-appointment dressings such as calcium hydroxide in the elimination of *E. faecalis* from deeper layers of dentin. Although some studies showed an enhanced ability to reduce viable cultures of *E. faecalis* using new irrigants such as MTAD™ or by combining calcium hydroxide with other medicaments or vehicles for delivery, these systems required extended treatment times and have not been tested in vivo, where conditions are ecologically diverse.

BACTERIAL CULTURING AND IDENTIFICATION OF BACTERIA IN INFECTED ROOT CANALS

Zielke et al. (171) compared the sensitivity of non-rereduced PRS medium (pre-reduced supplemented brain heart infusion) with TSB medium in 244 paired samples from 61 necrotic root canals at four different stages of the endodontic procedure. Teeth were sampled before and after preparation and at the second appointment immediately after removal of the temporary filling and cotton pellet and prior to obturation. No significant differences were found between non-rereduced PRS and TSB medium with 0.1% agar. The authors concluded the re-reduction step of PRS, used to remove oxygen entering the PRS medium at the time of insertion of the sample, improves the sensitivity of the medium and 7 days is needed for adequate incubation of bacterial samples.

Griffie et al. (172) compared bacterial growth in un-reduced TSB and reduced thioglycollate broth with an anaerobic sampling technique in samples from 17 necrotic teeth. The reduced thioglycollate medium was loosely capped and stored for at least 24 hours in nitrogen. The sampling media was placed in the endodontic access opening. The authors found 94% growth with reduced thioglycollate and 41% growth with un-reduced TSB. The authors concluded that TSB has agar present to help the growth of anaerobes but the oxygen must be removed by storing the medium in an oxygen-free environment or autoclaving prior to use.

Palmer et al. (173) evaluated in vitro paper point transfer of microorganisms from prepared root canals inoculated with dilutions of *S. faecalis* resistant to 2 mg/mL of streptomycin. The canals were cultured with dry sterile paper points and incubated in thioglycollate medium at 37°C for 7 days. All samples with bacteria exhibited growth by the seventh day. The authors found that fewer than 10 organisms gave positive readings in thioglycollate if the media were held and read for 7 days, and recommended culturing canals after introduction of sterile distilled water into the canal and evaluation of the culture at the obturation appointment.

Marshall and Savoie (174) compared the efficacy of endodontic culturing procedures using wet or dry paper points in 125 extracted human anterior teeth. Each tooth was reamed and filed through the apex to a #80 file and sterilized. Teeth in group 1 were then inoculated with *Serratia marcescens*, teeth in group 2 were inoculated with *S. albus*, teeth in group 3 were inoculated with *B. cereus*, teeth in group 4 were inoculated with a mixture of the three organisms above, and teeth in group 5 served as an un-treated control. Bacterial samples were then taken with sterile, dry, #6 paper points left in the

canal for 1 minute, with sterile #6 paper points following placement of 0.86% saline into the root canal, or with #6 paper points following placement of 0.1% peptone broth into the root canal. Dry paper points yielded positive growth in 36% of samples, paper points moistened with saline yielded growth in 93% of samples, and paper points moistened with peptone broth yielded growth in 63% of samples. The peptone method was significantly better than the dry-point method ($p < 0.01$), and the saline method was significantly better than the peptone method ($p < 0.01$). The authors concluded the added moisture in the canal provides a “pooling effect” that improves the collection of bacteria.

Carlsson and Sundqvist (175) evaluated 5 different methods of transport and cultivation of bacteria from infected root canals. The authors recovered bacteria from 29% of specimens when viability-preserving medium of the University of Goteborg No. IV (VMG IV) was used for transport and the specimens were subcultured in pre-reduced anaerobically sterilized (PRAS) medium or on the surface of blood agar in an anaerobic glove box. Bacteria were recovered from 49% of specimens when a PRAS-peptone-yeast extract broth was used for transport and the specimens were initially subcultured in the same broth. Bacteria were recovered from 58% of specimens when fluid thioglycollate medium USP was used both for transport and initial subculture, and from 47% of specimens when Clausen medium was used both for transport and initial subculture of the specimen. The authors advised fluid thioglycollate medium USP should be used for culturing in the dental office since it was as effective as PRAS medium and is available in screw-capped vials that require no special equipment in the dental office.

Pantera et al. (176) compared anaerobic culturing to indirect immunofluorescence for the detection of *Bacteroides* species in 30 extracted teeth and microbial samples from

82 patients. Anaerobic culturing detected *Bacteroides* in 27% of samples from extracted teeth and 20% from patient samples. Indirect immunofluorescence detected *Bacteroides* in 86% of samples from the same extracted teeth and 49% of the same patient samples. *Bacteroides intermedius* was identified from 11% of patient samples using anaerobic culture methods and from 43% of the same samples using indirect immunofluorescence. *Bacteroides gingivalis* was identified from 4% of patient samples using anaerobic culture methods and from 15% of the same samples using indirect immunofluorescence. *Bacteroides endodontalis* was identified from 1% of patient samples using anaerobic culture methods and from 16% of the same samples using indirect immunofluorescence. The sensitivity of indirect immunofluorescence compared to anaerobic culturing was 100%, while specificity for identifying particular species of *Bacteroides* ranged from 64% to 89%.

Möller (177) established the most commonly employed methods of microbial sampling from infected root canals and operative field decontamination in 1966. Six different sampling techniques were compared in 489 teeth in 373 patients. These techniques included: (1) a file was used in a moist or dry canal to scrape dentin filings from the canal wall; (2) a moist paper point was wiped along the canal wall in a moist or dry canal; (3) dry paper points were used in a fluid filled canal without prior pumping motion from a file for partial and (4) maximum removal of fluid in the canal; (5) dry paper points were used in a fluid filled canal with prior pumping motion using a file for partial and (6) maximum removal of fluid in the canal (PMR, pumping maximum removal, method). The PMR method was accomplished with the coarsest file that could be inserted into the entire length of the prepared part of the root canal and pumped

vigorously for 1 to 2 minutes. In root canals prepared 1 to 2 mm from the apex with variable bone rarefaction, the PMR method yielded growth in 41% of samples as compared to 13% with technique 1, 16% with technique 2, 18% with technique 3, 31% with technique 4, and 33% with technique 5. The PMR method used with sampling fluid (VGMA II) in the canal yielded significantly ($p < 0.001$) more samples with positive growth than any of the other techniques. In addition to sampling methods, the author also recommended transport and field decontamination protocols. In comparisons with reduced and non-reduced sampling and transport fluid, reduced VGMA II sampling fluid generated more growth than other sampling fluids. For field decontamination, the author recommended 30% hydrogen peroxide followed by 5% iodine tincture. This technique was compared to decontamination with Dakin's solution and was found to eliminate bacteria more effectively.

The techniques employed by Möller (177) have been adapted with the advent of modern microbiologic materials. Shuping et al. (30) and Card et al. (31) advocated the use of reduced dental transported fluid instead of VGMA II for use as a sampling fluid. Reduced transport fluid has the advantage of being a liquid instead of a gel and will maintain the viability of microorganisms up to 72 hours. Ng et al. (178) compared the effectiveness of 2.5% NaOCl and 10% iodine tincture for decontamination of the surfaces of teeth, rubber dam, and gasket material prior to culturing and molecular techniques for the detection of bacteria. Sixty-three teeth receiving root canal therapy were isolated with a rubber dam and Oraseal and the field of operation was disinfected with 30% hydrogen peroxide followed by either 2.5% NaOCl or 10% iodine tincture. Samples were taken before and after each decontamination step as well as before and after access

preparation. No significant differences were found between the NaOCl or iodine group using cultivation methods. However, when PCR was used as the bacterial detection method, samples from the tooth surface revealed significantly ($p = 0.010$) more bacterial growth with the iodine protocol (45%) compared with the NaOCl protocol (13%). The authors concluded NaOCl may provide more efficient decontamination of the operative field when molecular detection techniques are utilized.

Anaerobic sheep blood agar has been used to cultivate organisms commonly isolated from necrotic root canals, including *E. faecalis*. This enrichment medium is formed by the addition of citrated blood to tryptic soy agar, which permits differentiation of some species of bacteria based on characteristic hemolytic patterns and will support the growth of many fastidious organisms commonly isolated from cerebrospinal fluid, pleural fluid, sputum, and wound abscesses (148). Shuping et al. (30) used anaerobic sheep blood agar to cultivate organisms isolated from infected root canals following nickel-titanium rotary instrumentation with 1.25% NaOCl irrigation and medication with calcium hydroxide. The anaerobic sheep blood agar yielded growth on 98% of initial specimens, 62% of specimens following nickel-titanium rotary instrumentation, and in 7.5% of specimens following medication with calcium hydroxide. Waltimo et al. (161) used Brucella blood agar to compare in vitro the susceptibility of oral *Candida* species and *E. faecalis* to calcium hydroxide solutions at a pH of 12.4 for time periods ranging from 5 minutes to 6 hours. Colony-forming units were counted after 24 hours and 48 hours of incubation. Growth on the agar plates indicated equal resistance to calcium hydroxide for the strains of oral *Candida* and *E. faecalis* used in this study.

These studies collectively demonstrated the importance of using reduced transport fluid, moist paper points, enriched media such as blood agar, and anaerobic incubation for the cultivation of *E. faecalis* and other microorganisms commonly isolated from infected, necrotic root canals.

Traditional methods of microbial sampling for the detection and identification of specific organisms within infected root canals were shown by Reit et al. (179) and Molander et al. (180) to be less precise than molecular techniques. Molecular methods such as the polymerase chain reaction (PCR) have since gained popularity in the detection of the unique microflora within infected root canals. PCR is an in vitro method for the amplification of defined target DNA sequences present within a source of DNA, such as viable or dead bacterial cells. This results ultimately in the production of approximately 10^5 copies of the target DNA, which is visualized by agarose gel electrophoresis as a discrete band (181). Zambon and Haraszthy (182) demonstrated that PCR had a detection level of 10 bacterial cells. Ashimoto et al. (183) determined that 16S rRNA-based PCR was more accurate in detecting the presence of subgingival periodontal pathogens than culture procedures. Molander et al. (184) used specific PCR primers targeted against the 16S/23S rDNA intergenic region of *E. faecalis* and *E. faecium* and determined the sensitivity of the technique was high for the detection of both species from root canal samples. In a review of published epidemiological studies in the medical, dental, and biological literature, Siqueira et al. (185) further concluded that PCR is more sensitive and more accurate than traditional culture methods for the identification of particular bacteria in infected root canals.

PCR has been used to investigate the occurrence of *E. faecalis* within infected root canals. Siqueira et al. (186) used PCR to detect the presence of 19 bacteria from endodontically treated teeth with persistent periradicular lesions. *E. faecalis* was the most prevalent species isolated, found in 77% of samples.

Rôças et al. (187) used PCR to detect the presence of *E. faecalis* in infections of endodontic origin and to determine if this species is associated with different forms of periradicular disease. Eighty samples from infected root canals or periradicular abscesses were included in this study. Root canal samples were taken with paper points following surface disinfection and access into the pulp chamber, and abscess samples were taken by percutaneous aspiration. After sample processing, the supernatant was collected and used as the template for PCR amplification. Reference DNA from *E. faecalis* strain ATCC 29212 was used as a positive control for the primers. PCR amplification of the 16S rDNA sequence of *E. faecalis* was used to detect the presence of *E. faecalis*. The oligonucleotide species-specific forward primer used for *E. faecalis* was 5'-GTT TAT GCC GCA TGG CAT AAG AG-3', and the reverse primer was 5'-CCG TCA GGG GAC GTT CAG-3', producing a PCR amplicon of 310 bp. Five µL aliquots of the supernatant from the clinical samples were used as targets in the first PCR reaction using universal 16S rDNA primers. Afterward, 1 µL of the universal reaction was used as a template for the nested species-specific reaction. A second round of PCR amplification was used to assess the occurrence of *E. faecalis*. The PCR reaction was carried out in a thermal cycler with the following parameters: initial denaturation for 1 minute at 97°C, followed by 26 cycles of 45 seconds at 97°C, a primer annealing step at 55°C for 45 seconds, an extension step at 72°C for 1 minute, and a final step at 72°C for 4 minutes. The PCR

product was observed using ultraviolet illumination after electrophoresis on 1.5% agarose gel conducted at 4 V/cm in Tris-borate-EDTA buffer. Using this method of PCR, the authors detected *E. faecalis* in 18% of clinical specimens from teeth with primary apical periodontitis and 67% of clinical specimens from previously root-filled teeth.

Rôças et al. (188) used 16S rDNA-based PCR to identify the occurrence of 9 endodontic pathogens in previously root-filled teeth in a South Korean population. *Enterococcus faecalis* was the most frequently detected species, found in 64% of isolates. This study further demonstrated that *E. faecalis* is a frequent inhabitant of root canals with failing endodontic treatment. However, the authors stated that although *E. faecalis* was present in a majority of samples, PCR did not allow quantitative results, which made it difficult to ascertain if sufficient numbers of bacterial cells were present to participate in the infectious process. Nevertheless, the results of this study should not be dismissed, since the number of organisms required to perpetuate periradicular infection is still unknown.

Baumgartner et al. (189) used PCR to detect geographical differences of specific species of bacteria including *E. faecalis* in abscesses of endodontic origin obtained from Portland, OR and Rio de Janeiro, Brazil. Aseptic aspirates of 47 abscesses were obtained and 100 µL of each clinical sample were used for DNA extraction using a QIA Amp Tissue Kit following the method recommended by the manufacturer. The sequences of the 16s rRNA subunit of the target bacteria, including *E. faecalis*, were downloaded from GenBank. Variable regions of the highly homologous sequences were used to synthesize a pair of organism-specific oligonucleotide primers for each of the organisms tested. The specific primer used as the sense strand for *E. faecalis* was

CTTTCCTCCCGAGTGCTTGCACTCAATTGG and the anti-sense strand was CCGAAAGCGCCTTTCCTTACTCTTATG. The PCR reaction was carried out in a thermal cycler with the following parameters: initial denaturation for 1 minute at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 90 seconds at 72°C; final extension continued at 72°C for 10 minutes. The PCR product was observed using ultraviolet illumination after electrophoresis on 1.5% agarose gels containing ethidium bromide. Using this protocol for DNA isolation and for PCR, the authors isolated *E. faecalis* from 16% of specimens from Portland and 17% of samples from Rio de Janeiro. The difference was not statistically significant ($p > 0.05$). The authors concluded the use of primers highly specific for the organisms tested allows for precise detection of these organisms by PCR.

Siqueira et al. (157) used PCR and checkerboard DNA-DNA hybridization to detect the prevalence of *Actinomyces* species, streptococci, and *E. faecalis* in 53 infected teeth. The polymerase chain reaction used with ubiquitous primers detected the presence of bacteria in all 53 samples. Checkerboard DNA-DNA hybridization detected the presence of *Actinomyces* species in 9.4% of samples, streptococci in 23% of samples, and *E. faecalis* in 7.5% of samples.

These studies collectively demonstrated the enhanced ability of PCR over traditional cultivation methods to detect the presence of specific bacteria within infected root canals. However, PCR does not allow for quantitative comparisons of viable bacteria since DNA from both living and dead cells is amplified (188), obviating the need for alternative methods such as culturing to determine the exact number of intracanal bacteria present both pre- and post-instrumentation of infected root canals.

SODIUM HYPOCHLORITE AS AN IRRIGATING SOLUTION

Sodium hypochlorite has long been recognized as an effective irrigating solution. In 1915, Dakin (190) first used sodium hypochlorite as an antiseptic in open wounds. Walker (191) introduced sodium hypochlorite as a root canal irrigant in 1936. The author described double-strength chlorinated soda as an organic solvent and powerful germicide. In 1941, Grossman and Meiman (192) studied the tissue dissolving capability of various irrigating solutions. The authors found double-strength chlorinated soda to be the most effective solvent of pulp tissue.

Lewis (193) introduced the use of Clorox as a source of sodium hypochlorite in 1954. Until recently, Clorox contained 5.25% sodium hypochlorite, 4% sodium chloride, and 0.005 to 0.015% sodium hydroxide (194). Currently, Clorox (Ultra) comes in a concentration of 6% sodium hypochlorite and 94% inert ingredients. Sodium hypochlorite is manufactured by bubbling chlorine gas into sodium hydroxide, forming sodium hypochlorite and sodium chloride (195). Chlorine is the bactericidal agent in sodium hypochlorite. The molecule inhibits metabolic reactions of microbial enzymes by reacting with amino groups (NH_2) and sulfhydryl groups (SH) of bacterial amino acids (cystein).

The physio-chemical characteristics of sodium hypochlorite (NaOCl) are important in explaining its mechanism of action. The saponification, amino acid neutralization and chloramination reactions that occur when microorganisms and organic tissue are exposed to NaOCl are the cause for the antimicrobial and tissue dissolution properties. The antimicrobial activity is due to irreversible inactivation by hydroxyl ions and the chloramination reaction of bacterial essential enzymatic sites. The organic

dissolution action can be observed in the saponification reaction when sodium hypochlorite degrades lipids and fatty acids resulting in the formation of soap and glycerol (196).

Shih et al. (197) evaluated the ability of sodium hypochlorite to kill bacteria in the root canals of 120 extracted teeth. After making ten-fold serial dilutions, the authors found only full strength 5.25% sodium hypochlorite was an effective germicide.

Spångberg et al. (198) evaluated the cytotoxicity and bactericidal effect of a number of commonly used endodontic antimicrobial agents. Using cultures of HeLa and L cells, the authors found 5% sodium hypochlorite to be highly toxic and irritating to tissue and instead recommended the use of 0.5% sodium hypochlorite. The authors noted, however, the antibacterial properties of sodium hypochlorite were diminished at this concentration. The authors also recommended the use of 2% iodine potassium iodide in cases of persistent infection because of its low tissue toxicity and high antibacterial properties.

Rutberg et al. (199) also recommended the use of an iodophor as an irrigating solution. Using intradermal injections in rats to measure vascular leakage, the authors found 5% sodium hypochlorite caused severe tissue damage.

Ellerbruch and Murphy (200) tested the antibacterial activity of the vapors of various endodontic medicaments. The vapors of sodium hypochlorite and iodine potassium iodide were bactericidal and superior to all other agents tested except formocresol. The authors reported the antimicrobial activity of formocresol was greater at 24 hours than the other agents due to the bacteriostatic effects of its vapors.

Cunningham and Joseph (201) compared the in vitro bactericidal effect of 2.6% sodium hypochlorite at room temperature (32°C) and at body temperature (37°C). The authors recorded the initial time of zero growth of five test organisms subjected to the solutions and found the time required to eliminate bacterial growth was much less at body temperature.

Raphael et al. (194) conducted an in vitro investigation to determine the bactericidal efficacy of 5.25% sodium hypochlorite at varying temperatures. Instrumented and sterilized teeth were inoculated with various strains of bacteria and then irrigated with 5.25% sodium hypochlorite from 21°C to 37°C. The authors found no direct relationship between the temperature of the solution and bactericidal efficacy.

Harrison and Hand (202) studied the effects of organic matter and irrigant dilution on the antibacterial effects of 5.25% sodium hypochlorite. The authors exposed paper points contaminated with *Streptococcus faecalis* to test solutions for different time intervals and then cultured the points. The authors found that dilution of 5.25% sodium hypochlorite significantly decreased its antibacterial properties. However, the presence of organic matter did not affect its antibacterial effectiveness.

Smith and Wayman (203) used the same methods and materials as Harrison and Hand (202) to compare the antimicrobial effectiveness of 25% and 50% citric acid, 5.25% sodium hypochlorite, and sterile saline. Tests showed that citric acid at both concentrations was not as effective an antimicrobial agent as 5.25% sodium hypochlorite. Sterile saline had no antimicrobial properties.

Nikolaus et al. (204) also used the same method to compare the bactericidal effect of citric acid and sodium hypochlorite on anaerobic bacteria. The authors determined

that both 50% citric acid and 5.25% sodium hypochlorite were germicidal for all obligate anaerobes tested. Sterile saline had no germicidal activity on any of the bacteria tested.

Byström and Sundqvist (124) compared the antimicrobial effect of 0.5% sodium hypochlorite to saline in an in vivo study. Results showed that 0.5% sodium hypochlorite was more effective than saline in reducing the number of microorganisms. However, 20% of the canals tested were still positive for bacteria at the last appointment.

Byström and Sundqvist (125) also evaluated the antibacterial effect of 0.5% and 5% sodium hypochlorite during instrumentation of necrotic teeth in vivo. Bacterial culturing was performed before and after instrumentation for three appointments. Eighty percent of the bacterial strains isolated after the third appointment were anaerobic. No difference in antibacterial effectiveness was seen between the two solutions. The authors concluded the reaction of hypochlorite with the organic debris causes an inactivation of the sodium hypochlorite, reducing its antibacterial properties.

Foley et al. (205) studied the effectiveness of Clorox, Gly-Oxide, and saline against anaerobic bacteria. Combinations and dilutions of the various irrigating solutions were tested in both test tubes and inoculated, extracted teeth. Tube dilution studies showed that 5.25% Clorox and Gly-Oxide solutions were powerful antibacterial agents. However, dilution of the solutions diminished their antibacterial properties. In extracted teeth, Clorox was more effective than Gly-Oxide. Saline irrigation had no antimicrobial properties.

Harrison et al. (206) compared the antimicrobial properties of two different compositions of sodium hypochlorite in a tube dilution study. Absorbent paper points were contaminated with *S. faecalis* or *Candida albicans* and exposed to 5.25% or 2.62%

concentrations of "regular" or "fresh scent" sodium hypochlorite (Clorox) for periods ranging from 15 to 120 seconds. Results showed that formulary changes involved in the preparation of "fresh scent" sodium hypochlorite had no apparent effect on its antimicrobial properties, as both compositions proved equally effective against the test organisms at each concentration evaluated. No growth of *S. faecalis* was detected after a 45 second exposure to 5.25% NaOCl or a 60 second exposure to 2.62% NaOCl. *Candida albicans* was destroyed by all NaOCl solutions at fifteen-second intervals except 2.62% fresh scent Clorox.

Ohara et al. (207) determined the antibacterial effects of various endodontic irrigants against six selected anaerobic bacteria. The organisms were mixed separately with dilutions (1/10-1/400) of each of the irrigants in tubes containing fluid thioglycollate broth and allowed to remain in contact for specific time periods (1, 15, 30, 60 minutes, and 1 week) before being evaluated for growth. Of the six irrigants tested, chlorhexidine was the most effective antibacterial substance. Hydrogen peroxide, sodium hypochlorite and REDTA were less effective while the calcium hydroxide and saline solutions were totally ineffective.

Jeansonne and White (208) compared the antimicrobial activity of 2.0% chlorhexidine gluconate with 5.25% sodium hypochlorite in an in vitro root canal system. Freshly extracted human teeth with pulpal pathosis were instrumented using chlorhexidine, sodium hypochlorite, or saline as irrigants. Microbial samples were taken from the teeth immediately after accessing the canal, after instrumentation and irrigation, and after standing in an anaerobic atmosphere for 24 hours. Irrigation with chlorhexidine or sodium hypochlorite significantly reduced the numbers of post-irrigant positive

cultures and colony-forming units compared with saline-irrigated teeth. The number of post-irrigant positive cultures and the number of colony-forming units in positive cultures obtained from chlorhexidine-treated teeth were lower than the numbers obtained from sodium hypochlorite-treated teeth, but the differences were not statistically significant, either immediately after instrumentation or after 24 hours.

Kuruvilla and Kamath (209) evaluated the combined action of chlorhexidine gluconate and sodium hypochlorite within the root canal. Ten single-rooted necrotic anterior teeth were irrigated with 2.5% sodium hypochlorite alone, 0.2% chlorhexidine gluconate alone, 2.5% sodium hypochlorite and 0.2% chlorhexidine gluconate combined within the root canal, or 0.9% saline. Microbial samples for culture and Gram staining were taken before and following irrigation. The use of sodium hypochlorite and chlorhexidine gluconate combined within the root canal resulted in the greatest percent reduction of post-irrigant positive cultures. This may be due to formation of "chlorhexidine chloride," which increases the ionizing capacity of the chlorhexidine molecule. This reduction was significant compared to the use of sodium hypochlorite alone but not significant compared to the use of chlorhexidine gluconate alone.

Sen et al. (210) evaluated the antifungal properties of 0.12% chlorhexidine, 1% NaOCl, and 5% NaOCl. Root sections were enlarged and the smear layer was removed in half of the specimens. Each root canal was dispensed with an inoculum of *C. albicans*. After 10 days, the root sections were treated with 3 mL of either disinfectant solution for 1 minute, 5 minutes, 30 minutes, and 1 hour. In the presence of the smear layer, antifungal activity was observed only in the one-hour treatment group for all solutions. However, 5% NaOCl alone showed antifungal activity after 30 minutes in the absence of

the smear layer. The antimicrobial effectiveness of irrigating solutions should be re-evaluated, particularly in patients predisposed to oral candidiasis.

Overall, 5.25% sodium hypochlorite was proven to be an effective antibacterial agent when used as an irrigating solution. However, its antimicrobial effectiveness is decreased when it is diluted. Saline as an irrigating solution was shown to have no antibacterial effect.

ROOT CANAL ANATOMY OF MANDIBULAR MOLARS

Hess (10), in 1925, described the internal anatomy of 2,800 extracted human teeth by using corrosion preparations and histologic sections. The complex nature of root canal anatomy was described using unaided visual and microscopic analysis. The author concluded apical ramifications are often observed in the roots of upper molars and the mesial roots of lower first and second molars. The author also observed that in mesial roots of lower molars a fine connecting canal or fissure was frequently found between the two main canals.

Skidmore and Bjorndal (9) investigated the anatomy of mandibular molars by making plastic casts of 45 extracted teeth. The authors found that 59.5% of the mesial canals remained separate throughout the length of the canal, while 40.5% joined in a common apical foramen. In this study, 60% of the roots with two canals were found to have some form of transverse communication. This communication was most commonly observed in the apical third of the root. The authors noted that an operator attempting to remove the pulp tissue must be aware of these transverse connections.

Pineda and Kuttler (5) examined 859 extracted mandibular molars with a magnifying glass. The authors found ramifications (offshoots) of the main canal in the mesial root in 48.3% of first molars, 27.8% of second molars, and 19.0% of third molars. The authors also observed the majority of these ramifications were located in the apical third.

Cambruzzi and Marshall (211) studied canal anatomy by resecting 3 mm of the apical root of 108 extracted mandibular molars. A beveled cut was made to simulate an apicoectomy, and methylene blue dye was placed into the opened canals. The authors determined an isthmus joined the two mesial canals 60% of the time. Two mesial canals existed without an isthmus in only 18% of the cases.

Vertucci (11) used a hematoxylin dye and clearing technique to study the anatomy of 200 extracted mandibular first and second molars. In the mesial root, 63% of first molars and 31% of second molars were found to have a transverse communication. In approximately 75% of first and second molars this communication was found in the middle third of the root.

Manning et al. (12) studied the root canal anatomy of 149 mandibular second molars using a technique in which the pulp was removed, the canal space filled with black ink, and the roots demineralized and made transparent. Transverse anastomoses were found in 33% of roots, most commonly in the middle third of the root. Lateral canals were found in 72% of roots, most commonly in the apical third of the root. Apical deltas were found in 35% of the apices. The age of the patient and race affected canal shape, with more round canals present in patients over 35 years of age, and more C-shaped canals in Asians. The sex of the patient and the side of the mouth affected the

presence of apical deltas, with more found in males and on the left side. Single-rooted teeth had more complex root canal systems than two-rooted teeth, with more lateral canals, transverse anastomoses, apical deltas and C-shaped canals.

Yesilsoy et al. (13) observed 60 freshly extracted, mandibular first and second molars. Patency and the presence of two mesial canals were established with a #10 file. A vinyl polysiloxane impression material was injected into the pulp chambers and the teeth were centrifuged. The impressions were carefully removed and measured using a dissecting microscope and a transparent millimeter ruler. The ruler, at zero, placed at the mid-floor area of the impression and viewed from the mesial, measured (to the nearest 0.5 mm) the depth of the mesial groove between the mesiolingual and mesiobuccal canals. The average recorded depth was 1.0 mm. Some of the impressions had depths measuring 3.5 mm. This could be a significant space when considering the limitations of instrumentation techniques. The authors postulated this area may remain un-debrided after instrumentation thereby affecting the prognosis of treatment in both vital and necrotic teeth. The authors believed that modifications in access preparation and/or an increased emphasis on irrigation and intracanal medication may be needed.

Skillen (212), in 1932, stated that due to the complexities of form of the root canal, it is "practically impossible to remove the pulp in its entirety." The high percentage of fins, isthmuses, and accessory canals found by the above authors supports this statement.

STEP-BACK ROOT CANAL PREPARATION

Past studies (213-215) examined the effectiveness of biomechanical preparation in removing the contents of the root canal. However, all of these investigations reported that present techniques fall short of the goal of 100% canal cleanliness.

Coffae and Brilliant (213) and Klayman and Brilliant (214) showed the usefulness of the serialized (step-back) preparation technique in similar studies. Both investigations used the mesial roots of extracted mandibular molars. A grading scale was used to measure tissue contents at the 1 mm, 3 mm, and 5 mm apical levels. Coffae and Brilliant compared the serialization technique to a non-serialized instrumentation method. Klayman and Brilliant compared the serialization technique to the Giromatic instrumentation method. Both studies found the serialization technique was superior in tissue removal at all levels. Each study also concluded none of the methods effectively removes tissue from the isthmuses. Although the serialization technique removed tissue effectively, tissue and debris were still present within canals.

Walton (215) histologically compared the cleaning ability of filing, reaming, and step-back instrumentation techniques. The study used 91 canals from 52 vital teeth that were prepared in vivo, using 5.25% sodium hypochlorite for irrigation. The percentage of canal walls planed and the amount of predentin removed was evaluated. Step-back preparation was consistently superior to simple filing and reaming of the canal. The author also found that straight canals had consistently more planed walls than curved canals, regardless of the method used. No method was found to plane more than 85% of the canal walls.

The step-back method of hand instrumentation was shown to be an effective method of removing soft tissue from the root canal (213-215). However, all of these studies (213-215) demonstrated this method of instrumentation does not remove all debris from the complex root canal system.

ROTARY FILE AND CROWN-DOWN CANAL PREPARATION

Past studies (22-26) examined the effectiveness of biomechanical preparation in removing the contents of the root canal. However, all of these investigations reported that present techniques also fall short of the goal of 100% canal cleanliness.

Schafer and Zapke (22) investigated the cleaning effectiveness of automated and manual root canal instrumentation with the aid of a scanning electron microscope. Hand instrumentation was performed with K-Flexofiles used in a reaming motion and the step-back technique and with Hedström files used in a filing motion. Automated preparation was performed using torque-limited rotation with K-Flexofiles, as well as rotary nickel-titanium instruments (ProFile[®] system). After cracking the roots longitudinally (n =120), the amount of debris and smear layer was quantified on the basis of a numerical evaluation scale (1 through 5). Comparison of manual instrumentation with the automated KaVo-Endo Flash resulted in an equivalent degree of canal cleaning. Complete cleanliness was not achieved by any of the techniques or devices investigated. Best instrumentation results, especially in curved canals, were obtained with rotary ProFile[®] instruments.

Hulsmann et al. (23) compared several parameters of root canal preparation using two different rotary nickel-titanium instruments: Hero 642[®] (Micro-Mega, Besancon,

France) and Quantec[®] SC (Tycom, Irvine, CA, USA). Fifty extracted mandibular molars with root canal curvatures between 20° and 40° were imbedded into a muffle system. All root canals were prepared to a size #45 (Quantec[®] SC) or #40 (Hero 642[®]), respectively. The following parameters were evaluated: straightening of curved root canals, post-operative root canal diameter, safety issues (file fractures, perforations, apical blockages, loss of working length), cleaning ability, and working time. Both NiTi systems maintained curvature well; the mean degree of straightening was 2.3 degrees for Quantec[®] SC and 1.6 degrees for Hero 642[®]. Most procedural incidents occurred with Quantec[®] SC instruments (five fractures, three apical blockages, eight cases of loss of working length). Hero 642[®] preparations resulted in three blockages and one perforation. Following preparation with Hero 642[®], 63% of the root canals showed round, 24% oval, and 17% irregular diameters; Quantec[®] SC preparations resulted in a round diameter in 24%, oval shape in 29%, and irregular cross-section in 47% of the cases. Mean working time was shorter for Hero 642[®] (52 seconds) than for Quantec[®] (117 seconds). SEM was used to investigate cleanliness of the root canal walls using a five-score system for debris and smear layer. For debris, Hero 642[®] achieved better results (80% scores of (1)-clean root canal wall, only few small debris particles; and (2)-few small accumulations of debris) than Quantec[®] SC (76%). The results for smear layer were similar: cleaner root canal walls were found after preparation with Hero 642[®] (53% scores 1 and 2) than with Quantec[®] SC (41%). Both systems maintained original root canal curvature well and showed good cleaning ability. Quantec[®] SC showed deficiencies in terms of safety.

Versumer et al. (24) compared several parameters of root canal preparation using two different rotary nickel-titanium instruments: ProFile® .04 (Dentsply/ Maillefer, Ballaigues, Switzerland) and LightSpeed® (LightSpeed Technology Inc., San Antonio, TX). Fifty extracted mandibular molars with root canal curvatures between 20 degrees and 40 degrees were divided into two similar groups having equal mean curvatures. All root canals were prepared using ProFile® .04 or LightSpeed® Ni-Ti instruments to a size #45 following the manufacturer's instructions. The LightSpeed® system was used in a step-back technique. The ProFile® .04 instruments were used in a crown-down technique. The following parameters were evaluated: straightening of curved root canals (superimposition of pre- and post-operative radiographs), post-operative root canal diameter (superimposition of pre- and post-operative photographs of root canal cross-sections), safety issues (file fractures, perforations, apical blockages, loss of working length), cleaning ability (SEM evaluation of root canal walls using a five-score system for debris and smear layer), and working time. Both Ni-Ti systems maintained the original canal curvature well. The mean degree of straightening was less than 1 degree for both ProFile® .04 and LightSpeed® with no statistical significance between the groups. Most procedural incidents occurred with ProFile® .04 instruments (three fractures), while LightSpeed® preparation was completed without fracture of any instruments. Loss of working length, perforations, or apical blockage did not occur with either instrument. Following preparation with ProFile® .04, 64% of root canals had round, 30.7% oval, and 5.3% irregular cross-sections. LightSpeed® preparation resulted in a round cross-section in 41.3% of cases, an oval shape in 45.3% of cases, and an irregular cross-section in 13.3% of cases. No significant differences were found between

the two systems. LightSpeed® instruments enlarged the root canal more uniformly with no specimen showing 50% or more contact between pre- and post-operative diameter. Mean working time was significantly ($p = 0.02$) shorter for ProFile® .04 (105 seconds) than for LightSpeed® (140 seconds). For debris removal, LightSpeed® achieved better results (68% scores of 1 and 2) than ProFile® .04 (48.4% scores of 1 and 2), but no significant differences were found between the systems. The results for remaining smear layer were similar: the lowest amount of smear layer on the root canal walls was found after preparation with LightSpeed® (30.7% scores of 1 and 2) followed by ProFile® .04 (23.1% scores of 1 and 2). In the coronal third of the root canals LightSpeed® performed significantly better than ProFile® .04 ($p=0.029$). In the middle and apical thirds the differences were not significant.

Mayer et al. (25) evaluated debris and smear layer scores after two types of instruments manufactured from different alloys were used to ultrasonically activate irrigants during canal preparation. The influence of two rotary preparation techniques on cleanliness of the shaped canals was also studied. Apical stops were prepared to a size #45 in 42 single-canal extracted premolars and canines. Groups 1, 2, and 3 were prepared by ProFile® .04 (PF), while groups 4, 5, and 6 were prepared by LightSpeed® (LS). All groups were irrigated using 5.25% NaOCl and 17% EDTA. Irrigants were ultrasonically activated using a size #15 K-file in groups 2 and 5 and using a blunt flexible nickel-titanium wire in groups 3 and 6. Groups 2, 3, 5, and 6 received a one-minute ultrasonic treatment while the EDTA and NaOCl were left in the canals. Groups 1 and 4 served as negative controls. Roots were split and canal walls examined at 15x, 200x, and 400x magnification under SEM. Smear layer and debris scores were recorded

at 3, 6, and 9 mm levels using a 5-step scoring scale and a 200 μm grid. Although all groups had significantly higher smear layer and debris scores at the 3 mm level compared to the 9 mm level ($p < 0.05$), no significant differences (improvements) were recorded due to the ultrasonic energy transmitted by the two alloys. Ultrasonically activated irrigants did not reduce debris or smear layer scores. This finding was not influenced by the material or by the design of the instrument used to transmit ultrasonic activation.

Tan and Messer (26) compared the quality of apical enlargement of mesiobuccal canals of mandibular molars using conventional stainless steel hand files (K-files) and nickel-titanium (Ni-Ti) rotary instruments (LightSpeed[®]). Thirty freshly extracted mandibular molars were randomly assigned to three equal groups ($n = 10$ each group). The mesiobuccal canals were instrumented with K-files using a step-back technique without coronal flaring (control; group 1), K-files using a step-back technique after coronal flaring (group 2), and LightSpeed[®] (LS) instrumentation (group 3). Specific criteria were used for apical enlargement based on initial apical size. Canal cleanliness, canal transportation, and final canal shapes were determined histologically at 1 mm and 3 mm levels short of the working length. Canals were prepared to significantly larger sizes using LS instrumentation than with either hand instrumentation technique (15-17 ISO units, $p < 0.001$). LightSpeed[®] instrumentation allowed greater apical enlargement with significantly cleaner canals, less apical transportation, and better canal shape than both hand instrumentation groups at both levels ($p < 0.05$). None of the three techniques were totally effective in cleaning the apical canal space. The authors concluded that greater apical enlargement using LS rotary instruments is beneficial as an attempt to further debride the apical third region in mesiobuccal canals of mandibular molars. Instrument

designs, alloy properties, and canal curvature were important factors that determined the feasibility of greater apical enlargement in narrow canals.

Rotary instrumentation was shown to be an effective method of removing soft tissue from the root canal while also keeping with the curvature of the canal and having a uniform and rounded preparation (22-26). However, as the studies demonstrated, this method of instrumentation does not remove all of the debris from complex root canal systems.

ULTRASONICS IN ENDODONTICS

Richman (216) in 1957 first reported on the application of ultrasonics in endodontics. The author used a Cavitron® ultrasonic dental unit to clean, dry, and obturate root canals in over 50 cases. The investigator stated that since these cases were treated without untoward post-operative sequelae, the use of ultrasonics in root canal therapy held great promise.

In a series of articles published in the endodontic literature from 1976 to 1985, Martin and Cunningham (32-38, 217,218) reported on the use of ultrasound as a primary method of debridement in root canal therapy. These studies evaluated the efficacy of the Endosonic method, its ability to eliminate bacteria from the canal, its effect on extrusion, and its effect on post-operative pain.

In order to evaluate the efficacy of the use of ultrasound, Martin et al. (33) compared the ability of K-type files to remove dentin when powered by either hand manipulation or ultrasound. Two groups of seven standard canals in extracted teeth were each prepared by one operator using the two techniques. Using a Litton LT:200

(maximum output 50 watts at 18 KHz.) as an ultrasonic source and tap water as an irrigant, the authors found ultrasound was significantly superior to hand filing in total dentin removal. In a similar study using the same ultrasonic source, Martin et al. (34) compared the ability of diamond files and K-type files to remove dentin when powered by either ultrasound or hand manipulation. Using the same methods and materials as a previous study (33), the authors found that regardless of whether hand manipulation or ultrasound was used, the diamond files effectively removed more dentin than the K-type files. The authors also found that ultrasonically energized diamonds were significantly superior to hand manipulated diamonds. Cunningham et al. (35) compared ultrasonic filing to conventional hand filing for root canal debridement. The authors instrumented canals from extracted human teeth for 3 minutes using 2.5% sodium hypochlorite. After histologic preparation, sections from the 1 mm, 3 mm, and 5 mm apical levels were paired and subjectively evaluated for debris removal. Evaluating eleven pairs of teeth, ultrasound significantly removed more debris than hand instrumentation at all levels. In a similar study, Cunningham and Martin (36) used a scanning electron microscope to evaluate root canal debridement using hand methods or ultrasonic techniques. The authors found the ultrasound method produced cleaner canals at the mid-root and apical levels in all cases and reported the smear layer was "much less apparent" in the ultrasonically cleaned canals.

Cunningham et al. (217) evaluated the effect of Endosonic and hand filing techniques in reducing the number of bacterial spores in artificially contaminated root canals. Comparing different combinations of instrumentation techniques and irrigating solutions, the numbers of remaining organisms were measured. With saline as an

irrigating solution, the hand instrumentation group had a 62% reduction in microbial spores, while the ultrasonically instrumented group had an 86% reduction. When using sodium hypochlorite, the hand instrumentation group showed a 99.3% reduction, while the ultrasonically instrumented group showed a 99.8% reduction. This study showed the use of a sodium hypochlorite irrigating solution has more of an effect on bacterial reduction than the use of ultrasound.

Martin and Cunningham (37) measured the effect of the Endosonic system on the amount of root canal material extruded. Using 38 extracted teeth, the authors compared ultrasound preparation versus hand preparation by desiccating and weighing the amount of material extruded. The authors found the ultrasonically prepared teeth produced less extruded material whether instrumented short or past the apex. In both groups less extruded material was produced when instrumentation was short of the foramen.

Martin and Cunningham (38) evaluated the effect of Endosonic instrumentation on post-operative pain by comparing it against conventional root canal therapy. Each group had 164 patients, which were treated identically, except for the use of the Endosonic system. Pain was defined as a patient request for an analgesic other than aspirin. No significant difference was noted between the groups.

In an overview of their technique, Martin and Cunningham (218) stated that Endosonic root canal preparation was superior to hand preparation in mechanical and chemical debridement, disinfection, and final canal shaping. The ultrasonically energized file was reported to instrument the canal wall more efficiently and with less operator fatigue. The "ultrasonically activated" irrigant facilitated cleansing and disinfecting actions within the root canal system.

These studies by Martin and Cunningham (32-38, 217,218) laid the foundation for future investigations on the various aspects of the application of ultrasound in endodontics. These future studies involved the mechanisms of action of ultrasonic instrumentation, the use of ultrasound as a primary method of instrumentation, and the use of ultrasound as an adjunct to hand instrumentation. Each of these areas of investigation will be reviewed.

Mechanism of Action of Ultrasonic Instrumentation

Martin and Cunningham (218) attributed the success of ultrasonic instrumentation to the interaction of the ultrasonic energy and the irrigating solution. The authors called this interaction the synergistic system. The irrigating solution achieves its active biological-chemical effects when it undergoes ultrasonication. The authors defined the primary effects of ultrasound as cavitation and acoustic streaming. Transient cavitation occurs when the ultrasonic energy creates a bubble, which grows to a certain point and then collapses. This collapse creates a pressure-vacuum effect that cleans irregularities in canals and kills microorganisms. The oscillatory effect, which vigorously agitates the irrigating solution, is defined as resonant or stable cavitation. Combined with the effects of cavitation is a dispersal of physical energy that leads to physical acoustic streaming. Acoustic streaming (which will be described later) enhances cleansing and disinfection.

Martin and Cunningham (219) described the production of an "ultrasonic bath" in the root canal system. The authors utilized an Endosonic ultrasonic synergistic system, which employed a flow through, constant delivery of up to 45 mL of 2.5% sodium hypochlorite. Hydrodynamic action was created as this high volume of irrigant was continually aspirated. The authors indicated the continuous high-volume irrigation

interchange enhanced the ability to dissolve, flush, and remove debris. In essence, the root canal space and dentin walls prepared by the technique became an “ultrasonic bath” capable of cleaning the root canal with a high volume of continually replenished irrigant that loosened and removed debris. The authors concluded this multidimensional synergistic system results in a significantly cleaner root canal system.

In order to gain insight into the mechanisms involved in ultrasonic instrumentation, Ahmad et al. (45) investigated the phenomena of cavitation and acoustic streaming. In this study, the authors combined the phenomenon of resonant or stable cavitation, as described by Martin and Cunningham (218), with the phenomenon of acoustic streaming. These terms were combined because the rapid vortex-like motion associated with the vibrating file can also be associated with small gas bubbles set into oscillation by the fluctuating pressure field generated by the file. Transient cavitation was evaluated using a photometric-sensitive image intensification system. This detection system monitored the light produced by the violent collapse of cavitation bubbles. A rectangular container filled with methylene blue dye and a dispersed film of polystyrene spheres was used to detect acoustic streaming. These spheres were illuminated so that patterns of acoustic streaming could be detected. In this study, 40 extracted maxillary anterior teeth were divided into four groups and instrumented either by hand or ultrasonically (Cavi-Endo[®]), using either water or 2.5% sodium hypochlorite as an irrigating solution. The teeth were then split longitudinally and evaluated for the presence of a smear layer using a scanning electron microscope. The authors determined transient cavitation did not occur with the Cavi-Endo[®] unit and Endosonic files. However, cavitation was produced when a scaler tip was inserted into the unit. The

Endosonic files produced acoustic streaming. When the amount of remaining debris was evaluated, no statistically significant difference was found between ultrasonic and hand instrumentation when either water or sodium hypochlorite was used as an irrigating solution. The groups using the sodium hypochlorite always had less debris, regardless of the type of instrumentation. Ultrasound with either water or sodium hypochlorite significantly reduced the smear layer, but this reduction was of unequal distribution and thickness. The authors concluded that acoustic streaming is more important to cleaning than cavitation. The authors also concluded the recommended technique of ultrasonic instrumentation does not produce sufficient acoustic streaming to effectively clean the canal. Dampening of the files caused the limitation in the production of acoustic streaming in the constricted canal system.

Ahmad et al. (46) continued the investigation of ultrasonic debridement by examining acoustic streaming generated by the Cavi-Endo[®] unit. The authors defined acoustic streaming as the generation of time-independent, steady unidirectional circulation of fluid in the vicinity of a small vibrating object. Using the same method to detect acoustic streaming as described in the previous study (45), different size Endosonic files were studied at different power settings. The power generated by the files was estimated by measuring the transverse displacement amplitudes that were produced. Transverse displacement amplitude was defined as half of the total distance moved by the pinpoint of light that appeared as a thin transverse line when a file oscillated. Twenty extracted maxillary central incisors or canines were divided into 2 groups and instrumented either according to the manufacturer's instructions or with a modified technique. With the modified technique, a #15 Endosonic file was allowed to

freely vibrate at working length at a power setting of 2.5 for 5 minutes. The results showed that each file generated an acoustic streaming field comprised of a primary field consisting of rapidly moving eddies in which the fluid element oscillated about a mean position, and a superimposed secondary field consisting of patterns of relatively slow, time-independent flow. Approximately four clusters of eddies were generated by the #15 and #20 Endosonic files. In the primary field, the direction of rotation of the fluid elements in each eddy was opposite that of its immediate neighbor. The secondary field showed symmetrical longitudinal flows on both sides of the file. Fluid was generally transported from the apical to coronal end of the file. The streaming velocity was greatest at the apical and least at the coronal end of the file. Smaller files generated relatively greater acoustic streaming, the velocity of which increased with increased power. Canals instrumented with the modified method exhibited cleaner surfaces. The authors concluded the freely vibrating file produces hydrodynamic shear stresses large enough to remove debris and the smear layer from the walls of the root canal, resulting in enhanced cleansing action. This hydrodynamic shear stress was proportional to streaming velocity. Therefore, the authors deduced that since streaming velocity was highest at the apical tip of the file, a concentration of stresses in the vicinity of the tip facilitate debridement.

In another investigation into the mechanisms of ultrasound, Ahmad et al. (47) examined the effects of acoustic cavitation in debridement of root canals. In a preliminary study, a range of power settings in the Enac was evaluated to determine if cavitation was produced. Using a #15 file, the authors determined that cavitation could be produced at a minimum power setting of 3.5, and that a minimum displacement

amplitude of 135 microns was required for the file to oscillate freely (without binding) in a canal prepared apically to a #40 file. Twenty extracted maxillary canines were instrumented to a #40 file. In 10 teeth, a #15 file was allowed to freely vibrate at a setting of 3.5 for 5 minutes. A continuous flow of 2.5% sodium hypochlorite was provided during the period of instrumentation. In the other 10 teeth, a power setting of 1.0 was used. Cleanliness was determined by evaluating the remaining smear layer using a scanning electron microscope. Using this method of evaluation, the authors found no difference between the two groups. The authors also noted that cavitation may have produced small pits on the canal wall, and concluded that cavitation should not be regarded as an important mechanism in root canal debridement.

Walmsley (49) also investigated the mechanisms of ultrasound in root canal treatment. Agreeing with Ahmad et al. (47), the author concluded that cavitation has little if any bearing on the debridement activity of ultrasound. This conclusion was based on the postulation that although the displacement amplitudes of the vibrating file were adequate to produce cavitation, the streamlined shape of the Endosonic file was not conducive to generating a sound pressure field large enough to produce cavitation. The author also concluded that because of the transverse nature of the vibration pattern of the activated file, the effectiveness of ultrasonic instrumentation is limited by the dampening of the file against the root canal wall. Acoustic streaming is an effective mechanism in disrupting debris within the canals, but is reduced when loading occurs against canal walls. Also, the synergistic activity of ultrasound and the irrigating solution does not take place when the file is not allowed to vibrate freely.

Walmsley and Williams (220) further studied the effect of constraint on the oscillatory pattern of Endosonic files. The displacement amplitudes of various files were measured, using a Cavi-Endo[®] as an ultrasonic source. A model system utilizing pins in an acrylic block was used to simulate the effects of dampening which would occur during routine instrumentation. The constraining influence was greatest near the tip of the file, and increased if the file was angled. This constraint resulted in inefficiency of the ultrasonic system, especially in the apical third of a curved root canal. The authors recommended that a small diameter file should be used in a step-down technique to minimize the effects of constraint.

Lumley et al. (221) used slow setting plaster to demonstrate the activity of streaming around both ultrasonically and sonically activated files. For ultrasonic files, streaming occurred mainly in front of and behind the file, in contrast to the sonic file, where the plaster was distributed evenly around it. With both types of files most activity occurred around the file tip, and became reduced towards the driver. Streaming patterns associated with the ultrasonic device were dependent on the power setting of the instrument and whether the side of the file was constrained or lightly touched. The sonic device produced a large disturbance around the freely oscillating tip. Under load, this streaming occurred along the whole length of the file and was unaffected by constraint.

Ahmad et al. (222) found that ultrasonic files can generate acoustic streaming both in the free field and in the small channel. Higher velocity streaming was observed when smaller size files were employed and when the file was pre-curved. Light file-wall contact did not totally inhibit streaming while severe file-wall contact inhibited movement of the file and, as a result, no streaming was observed. The positions and

length scales of the streaming vortices appeared to be influenced by the presence of boundaries. In the free field, two rows of vortices were situated along the sides of the file; while in the small channel, the vortices were positioned above the surface of the file. These results indicated that it is possible for acoustic streaming to occur in a confined space, as in a root canal, provided that severe file-wall contact is avoided. The authors recommended that light filing or allowing the file to freely vibrate during some stage of treatment should be carried out in order to generate streaming in the root canal.

Ahmad et al. (223) studied the pattern of oscillation of a Piezon[®]-Master 400 (piezoelectric transducer) ultrasonic file in air and in water. Displacement amplitudes of the files were also measured. The authors observed the files vibrated such that a standing wave was formed and it exhibited points of maximum deflection (antinode) and points of minimum deflection (node) with the largest deflection occurring at the file tip. This pattern of oscillation was similar to that exhibited by the Cavi-Endo[®] file which employs a magnetostrictive transducer. However, the displacement amplitudes were much higher than those exhibited by the Cavi-Endo[®]. The authors hypothesized the 120° angle of the file holder, inherent in the Piezon[®]-Master 400 unit, and the more effective power transmission with the piezoelectric transducer may have contributed to the large amplitudes.

Ahmad et al. (224) also studied the Piezon[®]-Master 400 ultrasonic unit to determine variability in its power output when using different generators, transducers, and file holders. The displacement amplitude of the oscillating tip of the file in air was used as a measure of the power output. The results showed considerable variability in the power output of Piezon[®]-Master 400 ultrasonic files of similar size and length when

using different generators, transducers, and file holders. In consideration of this, the authors recommended that a calibration device be incorporated in the ultrasonic unit so the operator will have some knowledge of when the unit is working at its maximum efficiency.

In another study by Ahmad and Roy (225), the incidence of breakage of Piezon[®]-Master ultrasonic K-files was evaluated. Three groups of unused files were subjected to three treatments; namely, free vibration in air without irrigation, free vibration in the root canal while minimizing contact with the wall of the canal in the presence of irrigation, and light filing in the root canal with a free flow of irrigation. Cavitation produced by files in contact and free of contact with a glass surface was examined in order to observe the relationship between cavitation defects and breakage. In addition, the fractured and un-fractured files were examined under a scanning electron microscope for the presence of cavitation pits. The results indicated that more files broke in air. In water, a higher incidence of breakage occurred when files freely vibrated while no breakage occurred when the files were used in a light filing motion. All files generated cavitation, which resulted in pitting of their surfaces. However, the authors stated the pits were an unlikely cause of fracture. Fatigue cracks, possibly resulting from the manufacturing process, were observed at some of the corners of the cross sections of the fractured files and could be the main contributor to fracture.

Roy et al. (226) used sonoluminescence as an indicator of transient cavitation activity and photographic analysis was utilized as a means for detecting steady streaming, microstreaming, and stable cavitation with ultrasonic files. Measurements failed to indicate any strong correlation between registered driving power and the propensity to

produce transient cavitation. Files that were pitted or possessed salient edges were very effective at generating transient cavitation. When observed, transient cavitation activity generally occurred near the tip of a straight file, provided the wall loading did not inhibit file motion. In all cases studied, steady streaming and stable cavitation were observed to varying degrees, depending on the amount of file to wall contact. Stable cavitation was probably enhanced by the addition of moderate amounts of dissolved gas into the irrigant. Although the imposition of file-wall contact served to inhibit the production of transient cavitation, this action had relatively little effect on the ability of a file to produce a nominal level of streaming, microstreaming, and stable cavitation. Observations suggested that it was not prudent to ascribe enhanced cleaning effects to any one phenomenon, for it is likely that several factors are involved to varying degrees depending on the local conditions of application.

These studies (45-47,49,220-226) showed that dampening of the vibrating file limits the effectiveness of ultrasound. Therefore, an instrumentation method must be used which does not constrain the file in the canal.

Throughout the evolution of the uses of ultrasonics in endodontics, most technical changes have dealt primarily with ultrasonic generators. Two main types of generators are available that differ in mode of operation. The magnetostrictive generator utilizes the principle of magnetostriction in which certain materials expand and contract when placed in an alternating magnetic field (227). Alternating electrical energy from the ultrasonic generator is first converted into an alternating magnetic field through the use of a coil of wire (227). The alternating magnetic field is then used to induce mechanical vibrations at the ultrasonic frequency in resonant strips of nickel or other magnetostrictive material

that are attached to the surface to be vibrated (228). Because magnetostrictive materials behave identically to a magnetic field of either polarity, the frequency of the electrical energy applied to the transducer is half of the desired output frequency (227).

The piezoelectric generator converts alternating electrical energy directly to mechanical energy through the use of the piezoelectric effect, in which certain materials change dimension when an electric charge is applied to them (229). When electrical energy is applied to ceramic piezoelectric materials, a conversion and amplification of electrical energy into mechanical energy occurs that is then directly transmitted to the working tip (229). Piezoelectric generators are more efficient than magnetostrictive units because magnetostrictive units require two separate conversions of energy. Energy is lost each time one type of energy is converted into another (229). In magnetostrictive generators, electrical energy is converted to magnetic energy, which, in turn, is changed into mechanical energy. The piezoelectric units convert electrical energy directly into mechanical energy (229). This saved energy translates into added efficiency of the piezoelectric ultrasonic unit.

Ultrasound as a Primary Method of Instrumentation

Cameron (230) presented a clinical report on the use of ultrasound to clean root canals. A Cavitron® Model 700II was used with an endodontic insert and 3% sodium hypochlorite as an irrigating solution. Using hand files with the handle removed, the author found file breakage to be a problem. The author also reported the heat generated by the unit was capable of burning the patient's lip, even through the rubber dam.

Tauber et al. (39) evaluated the use of ultrasonically energized filing to serially instrument teeth. A Cavitron® unit with a spot-welded clip to hold conventional hand

files was used as an ultrasonic source. Each experimental group contained 11 extracted single-rooted teeth, and 0.5% sodium hypochlorite was used as an irrigating solution. Teeth were cracked longitudinally after instrumentation, and subjectively evaluated for remaining debris with a magnifying glass. Canals were divided into cervical, middle, and apical levels. Results showed no statistically significant difference in debris removal at any level. However, the ultrasonically instrumented teeth tended to contain less debris. The middle third of the canal was cleaner than the cervical or apical third. However, interjudge reliability was low among the five evaluators.

Cymerman, Jerome, and Moodnik (40) used a scanning electron microscope to compare hand instrumentation versus ultrasonic instrumentation. Twelve extracted single-rooted teeth were instrumented using either conventional hand instrumentation or ultrasonic instrumentation for 2 minutes using a Cavitron[®] unit with an endodontic insert. Normal saline was used as an irrigating solution. Teeth were prepared for SEM analysis, and photomicrographs from the coronal, middle and apical thirds were ordinally ranked according to remaining debris. Results showed that both methods produced root canal walls that were irregularly instrumented and smeared with tissue debris. No difference in canal wall appearance or amount of remaining debris was found between the two groups.

Langeland et al. (41) compared hand, ultrasonic, and sonic instrumentation in 65 extracted human teeth and 106 monkey incisors and canines in vivo. The Endosonic system with 1% sodium hypochlorite, and the Endostar 5 and Micro Mega 3000 with water were used as ultrasonic and sonic sources, respectively. One percent sodium hypochlorite was used with hand instrumentation. Slides were prepared for histologic analysis and sections were subjectively ranked according to the amount of remaining

debris. The authors found that each instrumentation method fully cleaned straight circular canals, but none of the methods completely cleaned curved or irregular canals. Therefore, the authors concluded root canal anatomy is more important in determining complete canal debridement than any cleaning device.

Chenail and Teplitsky (231) attempted to assess whether or not Endosonic instrumentation straightened curved root canals. A size #15 file was used in a Cavi-Endo® unit to instrument root canals in extracted teeth until a size #25 K-file would fit to working length. An overlay technique was then used with pre-operative and post-operative radiographs to evaluate the straightening of canal curvatures. Canal curvatures were straightened in 3 of 51 cases, with no measurable alterations in 94% of the cases. The authors concluded that Endosonics are safe to use in curved root canals.

Chenail and Teplitsky (232) repeated the same study, except size #20 and #25 Endosonic files were used instead of a size #15 Endosonic file. The authors found that all canals were straightened when using these files. Therefore, the authors recommended that size #15 Endosonic files should exclusively be used to instrument curved canals endosonically.

Pedicord et al. (42) compared hand versus ultrasonic instrumentation in relation to canal shape and instrumentation time. The mesial roots of 63 extracted mandibular molars were instrumented using either the step-back technique or the Endosonic system. Water was used as an irrigant and canals were instrumented apically to a size #25 file. Following instrumentation, roots were sectioned into thirds and evaluated for canal shape and location. Mean hand instrumentation time was significantly less than ultrasonic instrumentation time. Hand instrumentation produced a significantly better shape in the

middle and coronal levels. No significant difference in canal shape at the apical level was found with either hand or Endosonic techniques. Both techniques transported the canals equally toward the distal.

Kielt and Montgomery (233) used simulated canals in clear resin blocks to evaluate the effect of Endosonic instrumentation. Fifty resin blocks were divided equally and instrumented using either hand, sonic, or ultrasonic instrumentation. Sonic devices included either the Medidenta MM3000 or Syntex Endostar 5. Ultrasonic devices included either the Cavi-Endo[®] or the Osada Enac OE-2. All preparations were made to the size of a #35 K-file. Transportation measurements were made at the apical and middle portions of the preparations. The Medidenta and Caulk systems transported significantly less at the apex than the Syntex system and hand instrumentation. The Medidenta, Syntex, and hand instrumentation techniques transported significantly less in the middle portion than the Caulk and Osada systems. Overall, the Medidenta unit produced the least transportation.

Reynolds et al. (43) histologically compared the use of a step-back, sonic, and ultrasonic technique in small, curved root canals. Eighty root canals from extracted teeth were equally divided and instrumented using one of the techniques. The Endostar 5 was used as a sonic device, and the Cavi-Endo[®] and PZ KTec were used as ultrasonic devices. Following instrumentation, teeth were histologically prepared, and cross-sections examined for areas of remaining debris and predentin, the percentage of canal walls planed, and the percentage of increase in canal area. The authors found the hand instrumentation technique was more effective than either the sonic or ultrasonic techniques in increasing canal area, removing predentin and tissue debris, and planing

canal walls. These differences were noted primarily in the coronal and middle areas of the canal, with less difference between techniques in the apical level.

In a similar study, Stamos et al. (51) compared the debridement ability of hand, sonic, and ultrasonic instrumentation in the mesial roots of extracted mandibular molars. Five groups of ten teeth each were studied. Groups consisted of hand instrumentation with water irrigation, Endostar 5 sonic instrumentation with water irrigation, Enac ultrasonic instrumentation with water irrigation, and Cavi-Endo[®] ultrasonic instrumentation with either water or 2.6% sodium hypochlorite irrigation. After histologic preparation, the amount of remaining tissue debris was evaluated at the 1 mm and 3 mm levels using a polar planimeter. At the 1 mm level, the Cavi-Endo[®] group demonstrated significantly greater canal cleanliness than any other group except the Enac group. The Enac was significantly superior in canal debridement to the sonic or Cavi-Endo[®]/water group. At the 1 mm level, sonic instrumentation was not superior to hand instrumentation. At the 3 mm level, the Cavi-Endo[®] group showed the greatest level of canal and isthmus cleanliness, but the difference was not significant. Both sonic and ultrasonic instrumentation were significantly faster than hand instrumentation.

Lim et al. (234) studied the ability of hand, sonic, and ultrasonic instrumentation methods to clean root canals in extracted maxillary molars. A Micro-Mega 3000 was used as a sonic unit, an Enac OE-2 as an ultrasonic unit, and a step-back technique as the hand method. Water was used as an irrigating solution for all groups. Scanning electron microscope photomicrographs were subjectively graded for remaining tissue debris. The canals instrumented with the sonic device significantly removed more debris and smear layer than the other two methods.

Goldman et al. (44) compared three different methods of cleaning and shaping root canals in extracted maxillary central incisors. Each group of 20 teeth was instrumented with K-type and Hedström files, Burn's unfile, or ultrasonically with the Caulk Endosonic system. All groups used 5.25% sodium hypochlorite as an irrigating solution. One-half of each group was evaluated by injection with a silicone impression material and the other half was evaluated with a scanning electron microscope. The results showed that none of the methods produced a clean canal, and no statistically significant differences were found among the three groups. The authors observed the K-type/Hedström group prepared a smoother, tapered canal more often than the other two methods. The Burn's unfile produced a slightly better tapered apical preparation than the ultrasonic method.

Miserendino et al. (235) compared the cutting efficacy of sonic and ultrasonic instrumentation systems. Curved and straight artificial epoxy resin canals were instrumented using the Cavi-Endo[®] ultrasonic unit, the Endostar 5 sonic unit, the Micro-Mega 3000 sonic unit with either Rispisonic or Shaper files, or the Enac ultrasonic unit. In each group, #15 to #35 files were used in a sequential manner. Cutting efficacy was noted in terms of mean weight loss of each specimen. The authors concluded the Micro-Mega 3000, with either type of file, cut the resin blocks more efficiently than the other systems. The authors also found the cutting performance for most systems were equal in both straight and curved canals.

Calhoun and Montgomery (236) studied the effects of four instrumentation techniques on the root canal shape of the mesial roots of extracted mandibular molars. Acrylic blocks were constructed and 1.5 mm sections were made in such a manner the

blocks could be re-oriented after instrumentation. The four techniques consisted of step-back instrumentation with K-Flex files, balanced force instrumentation with Flex-R files, and Enac ultrasonic instrumentation with either Flex-R or Zipperer files. Although the results were not statistically significant, the Flex-R files with the balanced force technique tended to remain better centered in the canal. The total amount of dentin removed was significantly less with the step-back technique when compared to the other methods of instrumentation. At each canal level, all of the techniques tended to transport the original canal in the same direction. This transportation was mesial at the apical level, distal at the curve, and distal at the coronal level. The shape of balanced force instrumented canals was generally round, while canal shape in the other groups tended to be oval.

Yamaguchi et al. (237) used resin blocks and extracted anterior teeth to study the root canal cleansing and enlargement effects of ultrasonic instrumentation. Resin blocks with 10, 20, or 30 degree curvatures were filled with contrast medium and radiographs were taken pre- and post-operatively to determine the effect of instrumentation on canal shape. The extracted teeth were instrumented either by hand or ultrasonically and evaluated with a scanning electron microscope. The ultrasonic source was an Enac ultrasonic system. The authors found ultrasonic instrumentation produced an irregular shape a few millimeters from the apex, and recommended that when ultrasonic instrumentation is used, instruments should be pre-curved. The ultrasonic method was superior to hand instrumentation in the removal of debris from the canal.

Haikel and Allemann (238) also used a scanning electron microscope to evaluate the effectiveness of various methods of root canal preparation. The authors instrumented

140 extracted roots using either hand instrumentation with K- and H-files alternately, sonic preparation with the Sonic Air 3000 (using either Rispisonic and Helisonic files or Shaper files), and Canal Finder instrumentation. Hand preparation was similar to the Canal Finder. The surface condition of canals instrumented with the Sonic Air was better in the coronal and middle levels. Pulp residues were found in the apical level. The authors also noted the automated methods tended to straighten curved canals.

Bolanos et al. (239) compared instrumentation using engine and air driven methods to hand instrumentation. Twenty-eight extracted maxillary molars were categorized according to root curvature and instrumented using one of four methods: Endosonic Air 3000 with either Rispisonic or Shaper files, Giromatic with Rispi files, or hand instrumentation with K-Flex files. In straight canals, the Endosonic Air 3000 with Rispisonic files cleaned best at all levels. In curved canals, the sonic device with Shaper files cleaned the best in the apical level. The authors found the sonic system with either type of file performed better than the other two instrumentation techniques.

Baker et al. (240) used a scanning electron microscope to evaluate ultrasonic and hand instrumentation. Twenty-four extracted maxillary central incisors were instrumented using either hand or ultrasonic instrumentation with 2.6% sodium hypochlorite as an irrigating solution. Results showed no significant differences between ultrasonic and hand instrumentation at the apical and coronal levels. Hand instrumentation was significantly better at the mid-root level. A smear layer remained on canal walls after both methods of instrumentation.

Pugh et al. (241) compared the debridement efficacy of sonic and ultrasonic devices in 60 extracted molars. Sonic sources included the Sonic Air MM 3000 and the

Endostar 5. Ultrasonic devices were the Cavi-Endo® and Enac. After these devices were used, the final apical 2 mm were prepared with hand instruments. After instrumentation, canal shape and smoothness were evaluated by injecting impression material and decalcifying the specimens. Photographs of the specimens were subjectively graded using an ordinal scale. Results showed no significant difference between the groups.

Biffi and Rodrigues (242) compared hand instrumentation to ultrasonic instrumentation using a Profiendo unit as an ultrasonic source. Extracted maxillary bicuspid with two root canals were utilized in this investigation. Using 20 experimental teeth, each canal received one of the two methods of instrumentation using 0.5% sodium hypochlorite as an irrigating solution. Tissue debris was evaluated as either present or absent in the middle and apical thirds. The authors found that regardless of instrumentation technique, more tissue debris was left in the apical third than the middle third. The authors concluded the amount of debris remaining in the root canal depends more on the anatomy of the canal than the technique used.

Walker and del Rio (243) histologically evaluated hand, sonic, and ultrasonic instrumentation in curved canals. Mesial canals from 50 extracted mandibular molars were equally divided into 5 groups and instrumented using hand, ultrasonic, or sonic instrumentation. Ultrasonic devices included the Cavi-Endo® and Enac and sonic devices included the Medidenta and Endostar. Tap water was used as an irrigating solution in all cases. The authors found that all techniques were ineffective in planing all of the canal walls and in removing soft tissue from the main canal, isthmuses, fins, and multiple branches. Using the same 4 devices as Walker and del Rio (243), Yahya and El Deeb (244) compared the effects of ultrasonic and sonic instrumentation on various aspects of

root canal preparation. Instrumentation time, change in root canal angulation, and shape were evaluated in the mesial canals of 55 extracted mandibular molars. Sonic instrumentation with the MM 3000 and hand instrumentation were significantly faster than the other techniques. The only significant difference in angulation change existed between the MM 3000 (least) and the Enac (most). No significant differences were found between the groups in terms of working length change or change in mesiodistal canal location. The only significant difference in production of canal shape existed at the coronal level, where hand instrumentation produced the best shape. The authors noted this finding was probably due to the use of Gates-Glidden drills in the hand instrumentation group.

Loushine et al. (245) also evaluated root canal shape in mesial canals of mandibular molars following hand, sonic, or ultrasonic instrumentation. The Endostar 5 and the MM 3000 units were used as sonic sources and the Cavi-Endo[®] was used as an ultrasonic source. Buccal and lingual canals were alternately instrumented using the various techniques to allow direct comparison of root sections with a stereomicroscope. Hand instrumentation resulted in a significantly rounder shape than the other two methods. The MM 3000 produced significantly rounder shapes than the Cavi-Endo[®].

Tang and Stock (246) evaluated the effects of hand, sonic, and ultrasonic instrumentation on the shape of curved root canals. Extracted lateral incisors and premolars with moderate or severe canal curvature were filled with a radiocontrast medium. Canal shape was evaluated on pre- and post-operative photographic prints of the canals. A step-back technique was used as a hand method of instrumentation. A Cavi-Endo[®] unit was used as an ultrasonic source and a Micro-Mega 3000 was used as a

sonic unit. The authors found that as root curvature increased, the incidence of apical zip and elbow formation increased in the hand and sonic groups. However, the ultrasonic technique was found to produce smooth, progressively tapered preparations, regardless of root canal curvature. The authors also found that all three techniques removed more dentin from the convex aspect in the apical portion of the canal and more dentin from the concave aspect in the middle portion of the canal.

Ahmad and Pitt Ford (247) used macroradiography to compare the shape of canals following ultrasonic (Cavi-Endo[®]) and hand instrumentation. Extracted teeth with severely curved canals were instrumented using one of the two methods with 1% sodium hypochlorite as an irrigating solution. The use of subtraction radiography allowed pre- and post-instrumentation canal shapes to be evaluated on the same print. A digitizer was used to quantify the results. Results showed that both instrumentation techniques removed dentin unequally from canal walls, with more removal occurring coronally. No differences were observed in the time of instrumentation, the incidence of elbows, the distance of the elbow to the point of curvature, the change of width at the region of the elbow, and the apical transportation width. The authors concluded that ultrasonic files behave similarly to hand instruments when used in a filing action.

Ahmad and Pitt Ford (248) also compared the effects of instrumentation with the Cavi-Endo[®] and Enac units on the shape of root canals. Fifty simulated canals in clear resin blocks were divided equally and instrumented with either device using water as an irrigating solution. Both units were used at a power setting of 1. More resin was removed coronally than apically when evaluating photographic prints of the canals. Coronally, the Enac unit cut significantly better. However, the Enac group exhibited a

higher incidence of elbows, which occurred further apically than the elbows produced by the Cavi-Endo[®] group. The authors advised the apical areas of curved canals should be prepared only with a size #15 file, because larger less flexible files produced more elbows.

McCann et al. (249) evaluated the remaining dentin/cementum thickness in the mesial roots of extracted mandibular molars after hand instrumentation using a step-down technique, step-back technique, and ultrasonic instrumentation using the Endosonic system. Using a model system that could evaluate pre-operative and post-operative canal size on the same specimen, the authors found no significant difference between the two techniques. The authors determined that when using either method a danger of perforation exists at the distal aspect of the mesial root in an area 3.0-6.0 mm from the furcation.

Moorer and Wesselink (250) examined the effect of ultrasonics on the tissue dissolving effects of sodium hypochlorite. In these experiments, specimens of necrotic rabbit tissue were dissolved in different concentrations of sodium hypochlorite. The authors found that available chlorine was an important variable in tissue dissolution. Low concentrations of sodium hypochlorite (0.6% and 1.2%) were rapidly depleted of available chlorine. Three-percent sodium hypochlorite more effectively dissolved the tissue. When ultrasonic power was added to the tissue/sodium hypochlorite system, the solution was agitated violently, causing rapid dispersion and dissolution of tissue. The authors concluded the most important factor influencing tissue dissolution is the physical agitation caused by the ultrasonic energy.

Griffiths and Stock (251) also evaluated the relationship of ultrasound and irrigating solutions. Twenty-seven extracted teeth were divided into three groups and ultrasonically instrumented using water, Solvidont (bis-dequalinium acetate), or 2.6% sodium hypochlorite as an irrigating solution. A Cavi-Endo[®] was used as an ultrasonic source, and each tooth was instrumented apically to a size #25 or #30. Teeth were longitudinally sectioned, stained, and photographed. The authors found the sodium hypochlorite produced a cleaner canal than the other two solutions. No significant differences were found between water and Solvidont.

Cameron (252) investigated the synergistic relationship between ultrasound and sodium hypochlorite. Twenty-eight extracted teeth were instrumented to clinical standards with water used as an irrigating solution. Canals were given a final irrigation with 4% sodium hypochlorite, ultrasonically activated sodium hypochlorite, or ultrasonically activated water. Evaluation with a scanning electron microscope revealed that 4% sodium hypochlorite or ultrasound with water did not remove the smear layer inside the canal. However, 2% or 4% sodium hypochlorite did remove the smear layer when activated by ultrasound. Therefore, the authors concluded a clinically significant synergistic relationship does exist between ultrasound and sodium hypochlorite.

The studies of ultrasound as a primary method of instrumentation do not support the claim of Martin and Cunningham (218) that ultrasound removes more tissue from the canal than hand instrumentation. The majority of studies (39-41,44,229,241) found no difference in tissue removal between ultrasound and hand instrumentation. No difference was also found between the two instrumentation techniques when antibacterial effects

were evaluated (138,139). The overall performance of ultrasound as a primary method of instrumentation was not superior to hand instrumentation.

Ultrasound After Hand Instrumentation

Weller et al. (14) compared the efficacy of ultrasonics as a primary method of instrumentation and as an adjunct to hand instrumentation versus hand instrumentation alone. Thirty extracted single-rooted teeth and 30 resin blocks were pre-filled with radioactive gelatin and canal cleaning was based on the loss of radioactivity after instrumentation. The ultrasonic source consisted of a Cavitron® unit with an insert spot-welded to a 25 mm #15 stainless steel finger plugger. Sodium hypochlorite (5.25%) was used as an irrigating solution. In both the extracted teeth and the resin blocks, the authors determined the ultrasonic technique applied after completion of hand instrumentation and irrigation was significantly superior to either hand or ultrasonic instrumentation used alone. Therefore, the authors concluded that ultrasonic instrumentation is not an alternative to hand cleaning, but acts as an aid to increase debridement efficacy after hand instrumentation.

Goodman et al. (15) compared the effect of step-back preparation versus step-back/ultrasound instrumentation on tissue removal from the mesial root canals of 60 extracted human mandibular molars. Two operators performed identical procedures. The ultrasonic source consisted of a Buffalo piezoelectric dental unit with a #15 finger plugger soldered to the ultrasonic tip. Three-minutes of ultrasound were added per canal after completion of hand instrumentation. Continuous irrigation was provided utilizing 2.62% sodium hypochlorite. An additional 3 minutes of irrigation were applied to the hand instrumentation group to equalize the amount of irrigant used in each group. Tissue

removal in the canals and isthmuses was measured from transverse sections taken from the 1 mm and 3 mm levels, using a polar planimeter to measure the areas of the canals and remaining tissue debris. Results showed the step-back/ultrasonic technique cleaned canals at the 1 mm level and isthmuses at both levels more effectively than the step-back technique. No difference was found in canal cleanliness at the 3 mm level. Using hand instrumentation alone, a difference in canal cleanliness was observed between operators. However, in the step-back/ultrasound group, no difference was observed between operators.

In a similar study, Lev et al. (16) also compared the step-back technique to a step-back/ultrasound technique. This investigation used the same methods and materials as Goodman's (15) study, except that a Cavi-Endo[®] unit with a size #20 Endosonic file was used as an ultrasonic source. A one-minute ultrasound group was added in addition to the three-minute ultrasound group. Results showed no statistically significant difference between the groups at either the 1 mm or 3 mm level. However, the step-back/ultrasound three-minute group significantly cleaned the isthmuses at both levels more effectively than the other techniques.

Haidet et al. (17) also compared a step-back technique to a step-back/ultrasound technique in the mesial canals of human mandibular molars. This investigation was performed in vivo, with the teeth extracted immediately after instrumentation. A Cavi-Endo[®] unit with a #20 Endosonic file was used for 3 minutes in the step-back/ultrasound group. Equal amounts of irrigating solution (5.25% sodium hypochlorite) were used in both groups. At the 3 mm level, the authors found no difference in canal or isthmus cleaning between the 2 groups. At the 1 mm level, the step-back/ultrasound technique

was superior to the step-back technique in canal (99.6% versus 88%) and isthmus (86% versus 10%) cleanliness.

Metzler and Montgomery (18) obtained similar results when comparing the effectiveness of ultrasonic instrumentation and calcium hydroxide for the debridement of extracted human mandibular molars. Two-minutes of additional ultrasound were used following hand instrumentation (step-back technique). A Cavitron® unit with a Cavi-Endo® insert was used as an ultrasonic source. An intravenous bag connected to the insert in the handpiece supplied continuous irrigation with 2.6% sodium hypochlorite. In the second group, calcium hydroxide was allowed to remain in the canals for one week after hand instrumentation. Sections were similar to those used by Goodman et al. (15); however, an Olympus CUE-2 Image Analysis Program was used to evaluate the remaining tissue within the canal. Results showed that ultrasonics and calcium hydroxide were equally effective in debriding the root canal system, and that both techniques were significantly better than hand instrumentation alone in the isthmuses at the 1 mm level. The authors recommended that if multiple treatment appointments are required for root canal therapy, calcium hydroxide should be placed in the canals between appointments. If single visit treatment is used, ultrasonics should be used at the conclusion of hand instrumentation. However, if multiple appointments are used, ultrasonics could also be utilized prior to obturation.

Archer et al. (27) also compared the in vivo debridement efficacy of a step-back preparation versus a step-back/ultrasound preparation in the mesial root canals of vital mandibular molars. Group 1 consisted of 17 teeth prepared with a step-back technique using intermittent irrigation with 5.25% sodium hypochlorite. Group 2 consisted of 17

teeth prepared with a step-back technique as in group 1 followed by 3 minutes of ultrasonic instrumentation per canal utilizing a #15 Endosonic file in an Enac unit set at 3.5. An additional 6 mL/canal of 5.25% sodium hypochlorite was used during the ultrasonic preparation. Eight un-instrumented mandibular molars served as histologic controls. Following extraction and histologic preparation, 0.4 μ m cross-sections from the 1 mm to 3 mm apical levels of the canal and isthmuses were evaluated for percentage of tissue removal using an Olympus CUE-2 Image Analysis System. Factorial analysis of variance indicated canal and isthmus cleanliness values were significantly higher, at all 11 apical levels, with the ultrasonic technique. Sample values at the 1 mm, 2 mm, and 3 mm levels for the step-back and step-back/ultrasonic techniques, respectively, were: canal, 64% versus 92%, 81% versus 97%, and 90% versus 99.9%; and isthmus, 2% versus 46%, 15% versus 60%, and 16% versus 83%.

Cameron (21) studied 4% sodium hypochlorite and 15% ethylenediaminetetraacetic acid with centrimide (EDTAC) either alone or in conjunction as irrigants during hand instrumentation and ultrasonic irrigation of the root canal. Thirty-six extracted human teeth, each with a single, 21-25 mm long straight root were instrumented by hand through a clinical access cavity to a file size #40. One mL of the test irrigant was used after each instrument size. Canal debridement was completed with an intermittent flush irrigation technique with one or both of the test irrigants activated by ultrasound at a medium power output. The specimens were sectioned longitudinally, viewed in a scanning electron microscope, and scored for the presence or absence of debris and smear layer at levels less than 1 mm, 5 mm, and 10 mm from the apical seat. Under the conditions of this experiment the most effective regimen was irrigation with 1

mL EDTAC after each instrument size, followed by two thirty-second exposures to ultrasound plus EDTAC then four thirty-second exposures to ultrasound and 4% sodium hypochlorite. The specimens in this group were free of retained pulp tissue and superficial smear layer, had the lowest debris scores at the < 1 mm and 5 mm levels, and the lowest total debris score. All of the techniques tested produced smear-free canals at the 10 mm level.

Jensen et al. (19) compared the cleaning efficacy of passive ultrasonic activation with that of passive sonic activation after hand instrumentation. Sixty curved molar canals were hand-instrumented to size #35 and divided into three groups. Group 1 received no further treatment. Group 2 received 3 minutes of passive sonic activation. Group 3 received 3 minutes of passive ultrasonic activation. The roots were split and photomicrographs (x20) were made of the apical 6 mm of the canal. A transparent grid was placed over projected images, and the total number of squares covering the apical 6 mm of the canal space and the number of squares containing debris was counted. The mean debris scores were 31.6% for hand instrumentation only, 15.1% for the sonic group, and 16.7% for the ultrasonic group. The debris scores for the sonic and ultrasonic activation groups were significantly lower than the hand instrumentation only group ($p < 0.01$); however, no significant differences were found between the sonic and ultrasonic activation groups. Passive sonics after hand instrumentation produced a cleaner canal than hand instrumentation alone and was comparable to passive ultrasonics.

Mayer et al. (20) evaluated debris and smear layer scores after two types of instruments manufactured from different alloys (#15 K-file, NiTi Wire) were used to ultrasonically activate irrigants during canal preparation for 1 minute. The influence of

two rotary preparation techniques on cleanliness of the shaped canals was also studied. Apical stops were prepared to a size #45 in 42 single-canal extracted premolars and canines, which were divided into six equal groups. Groups 1, 2, and 3 were prepared by ProFile® .04 (PF), while groups 4, 5, and 6 were prepared by LightSpeed® (LS). All groups were irrigated using 5.25% NaOCl and 17% EDTA. Irrigants were ultrasonically activated using a size #15 stainless steel K-file in groups 2 and 5 and using a blunt flexible nickel-titanium wire in groups 3 and 6. Groups 1 and 4 served as negative controls. Roots were split and canal walls examined at 15x, 200x, and 400x magnification by SEM. Smear layer and debris scores were recorded at 3, 6, and 9 mm levels using a 5-step scoring scale and a 200 µm grid. Although all groups had significantly higher smear layer and debris scores at the 3 mm levels compared to the 9 mm levels ($p < 0.05$), no significant differences were recorded due to the ultrasonic energy transmitted by the two alloys. Ultrasonically activated irrigants did not reduce debris or smear layer scores. This finding was not influenced by the material or by the design of the instrument used to transmit ultrasonic activation.

Gutarts et al. (62) studied the effects of an ultrasonic irrigating needle as an adjunct to hand and rotary instrumentation in the mesial root canals of 36 mandibular molars with a clinical diagnosis of irreversible pulpitis. Teeth were placed into 2 groups. Group 1 consisted of the mesial root canals of mandibular molars prepared using hand and rotary instrumentation and intermittent irrigation with 6% NaOCl. Group 2 consisted of the mesial root canals of mandibular molars prepared using hand and rotary instrumentation with intermittent irrigation using 6% NaOCl followed by a one-minute ultrasonic application using an irrigating needle connected to a MiniEndo™ ultrasonic

unit and IV tubing through which passed 6% sodium hypochlorite. The irrigating needle was placed passively into each canal for 1 minute. Following access into the pulp chamber and electronic and radiographic verification of working length, crown-down instrumentation of the mesial root canals was performed as follows: (1) a #20 K-file was inserted to working length, (2) a #5 Gates-Glidden drill was used to facilitate instrumentation with rotary files, (3) ProFile® GT® rotary instruments in sizes #30/.10, #30/.08, #30/.06, and #30/.04 were taken to resistance, (4) ProFile® GT® orifice shapers in sizes #70/.12, #50/.12, and #35/.12 were used to further enlarge the coronal aspect of the canals, and (5) a #30 K-file was used to verify apical enlargement to a size #30. Two mL of 6% sodium hypochlorite were used as an irrigant after every third hand and rotary file. Ultrasonic instrumentation was then performed in a random set of these patients. Following instrumentation, each tooth was extracted and the apical 3 mm of each mesial root was sectioned and analyzed histologically using a Neurolucida Image Analysis Program version 5.0 for canal cleanliness. The authors found significantly ($p < 0.05$) less dentinal debris and pulp tissue remaining following the use of the ultrasonic irrigating needle regardless of canal type, canal curvature, or apical level. Canal isthmuses were also significantly cleaner ($p < 0.05$) following the use of the ultrasonic irrigating needle. The authors concluded the use of the ultrasonic irrigating needle with 6% NaOCl for 1 minute is clinically applicable and better able to debride the root canal system than hand and rotary instrumentation alone.

Many of these studies (14-21,27,62) showed that when ultrasound was added after hand instrumentation, at least one of the evaluated areas of the root canal was cleaner than when hand instrumentation was used alone.

SUMMARY

Taken collectively, these studies demonstrated the complexity of the root canal system and the relative ineffectiveness of current instrumentation procedures to completely debride and disinfect all areas of the root canal. The use of ultrasound following hand and rotary instrumentation may be a helpful adjunct in the removal of soft tissue and in the elimination of the microbial population within the root canal. However, further research is required to determine the efficacy and clinical applicability of various ultrasonic instrumentation procedures.

CHAPTER 3

MATERIALS AND METHODS

Selected portions of the following Materials and Methods were adapted from previous theses by Gutarts et al. (62), Goodman et al. (260), Lev et al. (261), Haidet et al. (262), and Archer et al. (263) from the Department of Endodontics at The Ohio State University.

Thirty-three volunteer adult patients presenting for endodontic treatment at The Ohio State University College of Dentistry were used in our study. All subjects were in good health as determined by a written health history and oral questioning. All female subjects were questioned regarding pregnancy or suspected pregnancy and were not allowed to participate if pregnant, suspecting a pregnancy, nursing, or were trying to become pregnant. The age and gender of each subject were recorded. Approval for this study was obtained from The Ohio State University Human Subjects Review Committee and written consent (Appendix C) was obtained from each participant. All subjects were required to complete a HIPPA release form (Appendix D) prior to enrollment in the study.

Qualifying subjects presented with a minimum 2 x 2 mm radiolucency on the mesial root periapex of a symptomatic or asymptomatic necrotic lower molar. A periapical film of the tooth, using a paralleling device (Rinn Corp., Elgin, IL) and Kodak

Ektaspeed film, and a radiographic exposure time ranging from 0.05 to 0.16 seconds at 7 Ma and 70 kVp, was used to determine the presence and size of the radiolucency.

Subjects included in our study had a mandibular molar with a clinical diagnosis of symptomatic or asymptomatic pulpal necrosis with acute or chronic periapical periodontitis. Tooth vitality was initially established with Green Endo-Ice[®] refrigerant spray (Hygenic, Akron, OH). The experimental tooth was dried with 2 x 2 inch cotton gauze and the refrigerant was sprayed onto a cotton pellet held by cotton forceps until the pellet was saturated. This pellet was then applied to the middle one-third of the buccal enamel surface (or lingual if the buccal was missing). The pellet was removed if the patient indicated a feeling of cold or pain by raising their hand. If the patient felt nothing and the refrigerant had evaporated from the pellet, a “no response” was recorded.

Vitality of each tooth was further analyzed with a Kerr Vitality Scanner (Kerr Dental, West Collins Orange, CA) digital electric pulp tester. After drying the tooth with 2 x 2 inch cotton gauze, a small amount of Colgate Total toothpaste (The Colgate-Palmolive Company, New York, NY) was used (enough to cover the electrode of the pulp tester) as a conducting medium between the pulp tester and tooth. The electrode was placed on sound enamel in the middle third of the buccal surface (or lingual if the buccal was missing) of the crown. The electrode was not placed on exposed dentin, cementum, or restorations. Pulp testing was started when the electrode made contact with the tooth and ceased when the patient indicated sensation in the tooth with a raised hand or if an 80/80 reading was reached. The digital value from the pulp tester was recorded. The patient was included in the study if an 80/80 reading was recorded for the experimental tooth. If an 80/80 was not reached, but the patient did not respond to the Green Endo-Ice[®] and had

a radiographic lesion present, the patient was temporarily included in the study until a definitive pulpal diagnosis was made upon access of the tooth. If any vital tissue was encountered upon access of the tooth the patient was excluded from the study. Only teeth with necrotic pulps were included in our study.

Radiographic findings were recorded by examination of the pre-treatment radiograph. The size of the radiolucency was recorded by measuring the greatest diameters along two perpendicular axes with a steel endodontic ruler. The first measurement was for the vertical axis and the second was for the horizontal axis. A history of previous endodontic therapy was also recorded. Subjects with a history of prior endodontic therapy on the involved tooth were not included in our study. The canal system was evaluated for the number of canals present radiographically and whether sclerosis of the canal(s) was present or absent. Subjects with extensive sclerosis of the root canal system were not included in our study.

Prior to injection, topical anesthesia (20% benzocaine gel; Patterson Brand Dental Supply, Inc., St. Paul, MN) was administered with a cotton swab in the area of the inferior alveolar nerve block injection. The cotton swab was left in place for thirty seconds and then removed. Two 1.8 mL cartridges of 2% lidocaine with 1:100,000 epinephrine (AstraZeneca LP, York, PA) were administered using an inferior alveolar nerve block and long buccal injection technique. The inferior alveolar and long buccal nerve blocks were administered using a 27-gauge short needle (Kendall Monoject, Mansfield, MA). The principal investigator gave all injections. The conventional inferior alveolar injection technique as described by Fischer (253) and modified by Jorgensen and Hayden (254) was used. The injection site was the soft tissue overlying

the medial surface of the ramus, lateral to the pterygomandibular raphe, at a height determined by the coronoid notch on the anterior border of the ramus. With the subject's mouth wide open, the thumb of the non-injecting hand was placed over the pterygomandibular triangle and then pulled laterally until the deepest depression in the anterior border of the ramus was felt. The first or second finger of the non-injecting hand was used to palpate the posterior portion of the ramus in order to find a slight depression. The line between the thumb and the finger was used to establish the vertical height of the injection site. The direction of needle insertion was from the contralateral mandibular premolars and directed parallel to the occlusal plane. The needle was advanced over a time period of ten seconds to the target site until bone was gently contacted at a depth of penetration of approximately 16 to 20 mm. After contact with bone was made, the needle was withdrawn 1 mm, aspiration performed, and the solution deposited at a rate of approximately 1 mL every 30 seconds. After all of the anesthetic solution was deposited the needle was fully withdrawn.

The conventional long buccal injection technique as described by Malamed (255) was also used. The area of insertion was the mucous membrane distal and buccal to the most distal mandibular molar, and the target was the buccal nerve as it passes over the anterior border of the ramus. With the subject's mouth wide open, the index finger of the non-injecting hand was used to pull the buccal soft tissues in the area of injection laterally to improve visibility. The syringe was directed toward the injection site with the bevel facing down toward the bone and the syringe aligned parallel and buccal to the occlusal plane on the side of the injection. The mucous membrane was penetrated at the injection site, distal and buccal to the last molar. The needle was advanced slowly until

the mucoperiosteum was gently contacted. After contact with bone was made and aspiration performed, the solution was deposited at a rate of approximately 0.5 mL/15 seconds. After all of the anesthetic solution was deposited the needle was fully withdrawn. Approximately 3.1 mL of anesthetic was used for the inferior alveolar injection and approximately 0.5 mL of anesthetic was used for the long buccal injection.

The experimental teeth were randomly divided into two groups. Group 1 consisted of a parallel control group of 17 mesial root canals of mandibular molars prepared in vivo with a hand and rotary file technique using intermittent irrigation with 6.0% sodium hypochlorite. Group 2 consisted of 16 mesial root canals of mandibular molars prepared in vivo with a hand and rotary file technique using intermittent irrigation with 6.0% sodium hypochlorite followed by 1 minute of ultrasonic irrigation per canal utilizing a 25-gauge needle activated by an ultrasonic unit (MiniEndo™, Analytic EIE Inc., San Diego, CA) set at full power.

Prior to initiation of the study, random six-digit numbers were recorded on a master code list corresponding to the experimental groups. The numbers "1" or "2," representing the experimental groups, were recorded on an equal number of pieces of paper. One piece of paper was sealed in an envelope with the random code number recorded on the outside. The envelopes were shuffled and then placed in a cardboard box. A sealed envelope was selected and opened after the hand and rotary instrumentation was completed for each experimental tooth. Since the determination of experimental group assignment was made after the hand and rotary preparation was completed, operator bias was eliminated. Both canals of the mesial root of the experimental tooth received the same treatment. After fifteen subjects from each group

were completed, one envelope from Group 1 was paired with an envelope from the other experimental group. For each subsequent subject, one of these envelopes was chosen after the hand and rotary instrumentation procedure was completed. This ensured equal distribution of teeth between the two groups. The envelopes were used later to store the radiographs exposed for each experimental tooth. The random number on the outside of the envelope identified the radiographs.

Experimental teeth were isolated with a rubber dam. After the patient was anesthetized and the rubber dam was in place, the tooth, adjacent rubber dam, and rubber dam clamp were cleansed with 30% hydrogen peroxide (Mallinckrodt, Paris, KY) until no further effervescence of the peroxide occurred. The 30% hydrogen peroxide was vigorously scrubbed on all surfaces for a period of 30 seconds using sterile cotton swabs saturated with the solution. If bubbling was incessant, Cavit[®] (3M ESPE, Germany) was placed around the neck of the tooth and the process repeated. The tooth, rubber dam, rubber dam clamp, and Cavit[®] were then disinfected with Povidone-Iodine Swabsticks (Medline Industries, Mundelein, IL). The Swabsticks were applied to all surfaces for 30 seconds and the povidone-iodine was allowed to dry for 2 minutes.

A standard access opening was made using a #4 round bur in a high-speed handpiece. Canal orifices were located and the absence of pulpal hemorrhage was noted as confirmation of pulpal necrosis. After the access opening was made and the canals located, sterile orifice openers (ProFile[®] GT[®] files sizes #70/.12, #50/.12, #35/.12, Dentsply Tulsa Dental, Tulsa, OK) were utilized for coronal flaring of the distal canal. Another set of identical sterile orifice openers were then utilized for coronal flaring of the mesial canals followed by 2 mL of sterile 0.9% sodium chloride irrigation (Baxter

Healthcare Corp., Deerfield, IL) of the pulp chamber only. Cavit[®] was placed in the access opening to occlude the distal canal during instrumentation and bacterial sampling of the mesiobuccal and mesiolingual canals. Approximately 2 mL of sterile 0.9% sodium chloride irrigation were again used to flush any debris from the pulp chamber. The pulp chamber was then dried with sterile cotton pellets, and 0.02 mL of liquid dental transport fluid (LDT, Anaerobe Systems, Morgan Hill, CA) were placed in the mesiobuccal and mesiolingual canals with a sterile tuberculin syringe. A provisional working length was determined by measuring the approximate canal length on the pre-operative radiograph. The canal contents were dispersed into the LDT with sterile #10-20 K-type hand files (Dentsply Maillefer, Tulsa, OK) placed to within 1 mm of the estimated working length. Sterile x-fine to fine paper points were then placed in the mesiobuccal and mesiolingual canals as close as possible to the estimated working length and transferred to a vial containing 1.0 mL of LDT. This constituted the initial bacterial sample (S1).

GROUP 1 - ROOT CANAL PREPARATION

K-type hand files and rotary ProFile[®] GT[®] files (Dentsply Tulsa Dental, Tulsa, OK) were used for canal preparation. A #10 K-file was placed into each mesial canal using a one-quarter turn, push-pull motion, until the end of the file was approximately 1 mm from the radiographic apex. This was used for initial canal exploration, and was followed by a #15 K-file placed to approximately 1 mm from the radiographic apex. The Root ZX[®] Apex Locator (J. Morita, Irvine, CA) was then used with a #10 K-file to determine the working length approximately 0.5 mm (apical constriction) from the actual apex. A radiograph was exposed with a slight mesial angulation (approximately 15°

from perpendicular) with a #15 K-file in each mesial canal to determine the position of each file, and to confirm the working length determined by the Root ZX[®] Apex Locator. Files were removed, measured with a steel endodontic ruler, and the lengths recorded.

After working length was established for each mesial canal and each canal was filed to a size #20 hand file, a crown-down technique was used to enlarge the coronal portion of the canal, then the mid-root, and finally the apical third with rotary files. Each canal was irrigated with 2 mL of 6.0% sodium hypochlorite (The Clorox Company, Oakland, CA) following the use of every third hand and rotary file. The sodium hypochlorite was delivered using a 25-gauge, 5/8-inch needle with a Luer-Lok attachment connected to a 20 mL disposable, plastic Luer-Lok syringe. Each rotary file was used with Glyde (Jordco Inc., Beaverton, OR) lubricant during instrumentation of each canal. The following instrumentation sequence was used:

- Step #1: #10 K-file (canal exploration)
- Step #2: #15 K-file
- Step #3: #10 K-file used with Root ZX[®] (establish working length)
- Step #4: #15 K-file, radiograph taken (confirm working length)
- Step #5: #20 K-file
- Step #6: #5 Gates-Glidden
- Step #5: ProFile[®] GT[®] (#30/.10)
- Step #6: ProFile[®] GT[®] (#30/.08)
- Step #7: ProFile[®] GT[®] (#30/.06)
- Step #8: ProFile[®] GT[®] (#30/.04)
- Step #9: ProFile[®] GT[®] (#70/.12)

- Step #10: ProFile® GT® (#50/.12)
- Step #11: ProFile® GT® (#35/.12)
- Step #13: #30 K-file, to confirm apical portion enlargement to a size #30.

The largest size K-file (#30) was inserted to ensure the initial working length was maintained and a radiograph was exposed to verify its location. If the placement of the final file was short of the calculated working length, an alternating procedure (212,256,257) was used with a #10 through #25 K-file to get the final K-file to the calculated working length. After completion of hand and rotary instrumentation, an additional 15 mL of 6.0% sodium hypochlorite delivered from a 20 mL disposable, plastic Luer-Lok syringe with an attached 25-gauge, 5/8-inch needle was used to irrigate each mesial canal.

Following hand and rotary instrumentation of the mesial canals, a second bacterial sample was collected. This sample was collected using the pumping maximum removal (PMR) method originally described by Möller (177), with the exception that LDT was used as the transport medium instead of VGMA II. After instrumentation and final irrigation with 6.0% NaOCl, each mesial canal was dried with sterile medium and coarse paper points, flushed with 2 mL of 5.0% sodium thiosulfate (Red Bird Service, Osgood, IN) for 1 minute, and rinsed with 2 mL of sterile 0.9% sodium chloride irrigation. The canals were again dried with sterile medium and coarse paper points and 0.02 mL of LDT were placed in the mesiobuccal and mesiolingual canals using a sterile tuberculin syringe. A sterile K-file equal in size to the master apical file was placed to working length and pumped five times with minimal reaming motion to disperse the canal contents into the LDT. Sterile medium and coarse paper points were then used to absorb the canal

contents and the paper points were transferred to a vial containing 1.0 mL of LDT. This constituted the second bacterial sample (S2).

Following collection of S2, three subjects in Group 1 received an additional 15 mL of 6.0% sodium hypochlorite in each mesial canal delivered from a 20 mL disposable, plastic Luer-Lok syringe with an attached 25-gauge, 5/8 inch needle, as a control procedure. The total amount of 6.0% sodium hypochlorite used in each mesial canal in these subjects following hand and rotary preparation and after additional irrigation was 30 mL. After irrigation, each mesial canal was dried with sterile medium and coarse paper points, flushed with 2 mL of 5.0% sodium thiosulfate for 1 minute, and rinsed with 2 mL of sterile 0.9% sodium chloride solution. The canals were again dried with sterile medium and coarse paper points and 0.02 mL of LDT were placed in the mesiobuccal and mesiolingual canals using a sterile tuberculin syringe. A sterile K-file equal in size to the master apical file was placed to working length and pumped five times with minimal reaming motion to disperse the canal contents into the LDT. Sterile medium and coarse paper points were then used to absorb the canal contents and the paper points were transferred to a vial containing 1.0 mL of LDT. This constituted the third bacterial sample (S3).

Complete instrumentation of the distal canal followed the second or third sampling procedure (the distal canal was not included in the bacterial sampling procedure), a temporary restorative material (Cavit[®] or IRM[®]) was placed in the access opening, and the subject was given post-operative instructions as well as an appointment to have the root canal therapy completed.

GROUP 2 - ROOT CANAL PREPARATION PLUS ONE MINUTE OF ULTRASONIC IRRIGATION

The teeth in this group were prepared in an identical method to those teeth in Group 1 with the addition of an ultrasonically energized needle used after completion of the hand and rotary preparation. Initial bacterial sampling (S1) as well as bacterial sampling following completion of hand and rotary instrumentation (S2) was performed in an identical method to those teeth in Group 1. The MiniEndo™ unit was used for ultrasonic irrigation (Analytic EIE Inc., San Diego, CA). This unit is a piezoelectric instrument, which is powered by a standard electrical power source and does not require a coolant.

The power adjustment on the unit was set at full power. A new 1.5 inch, 25-gauge, sterile irrigating needle (Becton Dickinson & Company, Franklin Lakes, NJ) was used for each tooth. Each needle was inserted through the rear aperture of the shaft of the ultrasonic tip device and connected to the MiniEndo™ handpiece. The needle was at a 45° angle to the long axis of the ultrasonic handpiece. The needle was directed through the bore of the shaft and out the end where it was tightened in place by a screw-on hub so that 15-20 mm of the needle was exposed. Luer-Lok intravenous tubing connected the needle in the handpiece to a 20 mL syringe containing 20 mL of 6.0% sodium hypochlorite (Figure 1).

Upon completion of hand and rotary preparation and after the second bacterial sample was obtained, as described for Group 1, each canal was filled with 1 mL of 6.0% sodium hypochlorite using the needle and syringe previously described. Prior to activation of the ultrasonic unit, a sterile rubber stop was placed on the irrigating needle

and the needle inserted into the canal to a point just short of binding. The rubber stop was moved to the reference point previously established for canal preparation, the irrigating needle removed, and the distance from the tip of the needle to the base of the rubber stop was measured using a steel endodontic ruler in order to determine the depth of penetration of the irrigating needle into the canal. High-speed suction, using a surgical aspirating tip, was placed at the distal aspect of the tooth and maintained at this position during irrigation. Keeping the aspirating tip in this position allowed the pulp chamber to remain full of irrigant and prevented the overflow of excess solution out of the tooth. The ultrasonic needle, connected to the handpiece, was placed in the canal to the measured depth previously recorded (Figure 2). Upon activation, the needle was moved passively in an up-and-down motion to ensure it did not bind within the root canal. The energized ultrasonic needle was used continuously for 1 minute in each canal. Six percent sodium hypochlorite was delivered from a 20 mL syringe at a rate of 15 mL/minute through the intravenous tubing connected to the ultrasonic needle. This procedure was then repeated for the other mesial canal. The same ultrasonic needle was used in both mesial canals and then discarded. The total amount of 6.0% sodium hypochlorite used in each mesial canal following hand and rotary instrumentation and after ultrasonic instrumentation was 30 mL. Following ultrasonic irrigation, a third bacterial sample (S3) was collected as previously described for all S2 samples.

Complete instrumentation of the distal canal followed the third sampling procedure (the distal canal was not included in the bacterial sampling procedure), a temporary restorative material (Cavit[®] or IRM[®]) was placed in the access opening, and

the subject was given post-operative instructions as well as an appointment to have the root canal therapy completed.

MICROBIOLOGIC PREPARATION

Each bacterial sample was transported in LDT to a dental microbiology laboratory at The Ohio State University for quantification of colony forming units. Twenty percent ethyl alcohol was used to disinfect the bench top prior to specimen preparation. A propane flame was maintained in order to disinfect the top of each screw-top vial containing the bacterial samples prior to dilution. A negative control for unwanted bacterial growth during the microbiologic preparation of samples was created by placing sterile paper points in LDT, making serial dilutions of the control sample, and plating these dilutions along with the experimental bacterial samples. Each bacterial sample was vortexed for 30 seconds to disperse the contents within the LDT vial. Prior to dilution, 0.25 mL of the original sample were streaked on an anaerobic sheep blood agar plate (Brucella blood agar, Anaerobe Systems, Morgan Hill, CA). A streak plate technique was used for isolation of individual colonies of bacteria. The microbial mixture was transferred to the center of an anaerobic sheep blood agar plate utilizing a P-1000 Pipetman[®] micropipette with sterile pipette tips (Fisher Scientific, Pittsburgh, PA) and then streaked over the surface with a sterile 60 mm cell spreader (Fisher Scientific, Pittsburgh, PA) while spinning the agar plate in a clockwise manner on a plate-spinner. Ten-, 100-, and 1000-fold serial dilutions of the original sample were then prepared in the following manner: (1) the original sample was vortexed for 30 seconds; (2) 30 μ L of the original sample were transferred aseptically with a P-200 Pipetman[®] micropipette (Rainin, Oakland, CA) to 270 μ L of LDT in a sterile microcentrifuge tube (Fischer

Scientific, Pittsburgh, PA) to create a 1/10 dilution and the tube vortexed; (3) 30 μ L of the 1/10 dilution were transferred aseptically to 270 μ L of LDT in a sterile microcentrifuge tube to create a 1/100 dilution and the tube vortexed; and (4) 30 μ L of the 1/100 dilution were transferred aseptically to 270 μ L of LDT in a sterile microcentrifuge tube to create a 1/1000 dilution and the tube vortexed. Aliquots of 0.25 mL of each diluted sample were then transferred to an anaerobic sheep blood agar plate using a P-1000 micropipette with sterile pipette tips and then streaked over the surface of the agar with a cell spreader while spinning the agar plate in a clockwise manner on a plate-spinner. The agar plates were labeled by random number, sample (S1, S2, or S3), dilution factor and date, and then incubated at 37°C for 7 days in an anaerobic chamber containing 5% carbon dioxide, 10% hydrogen, and 85% nitrogen.

METHOD OF EVALUATION

The principal investigator prior to decoding the random numbers evaluated colony growth on the agar plates. This ensured the evaluator was blinded to the instrumentation technique utilized on each specimen. Individual colony forming units were counted following the seven-day anaerobic incubation period. Each agar plate was placed on a light source to provide enhanced illumination for colony counting. Single, distinct colonies were identified and counted visually using a microscope at 10x magnification. Each counted colony was marked by placing a dot with a black permanent marker on the surface of the agar next to each colony to ensure the same colony was not counted twice. Only agar plates with colony forming units numbering less than 250 were counted. The number of colony forming units per sample was calculated using the formula:

$$(\text{\#CFU} \times 4) \times 10^{|y|+1} \times 1 \text{ mL LDT/per sample vial} = \text{\#CFU/sample.}$$

In this formula, y is equivalent to the dilution factor used in a specimen. For example, in a 10-fold (1/10) dilution, $y = |-1| = 1$ (30). A $\log_{10}$ transformation of each recorded S1 and S2 CFU count was used to normalize the data prior to statistical evaluation.

Initial radiographs of the experimental teeth were analyzed using Schneider's method (259) to determine the curvature of the mesial root. These radiographs were projected on white paper using a standard slide projector. A straight line was drawn through the coronal portion of the mesial canal and through the long axis of the root. A second line connected the apical foramen to the point on the first line where the canal began to deviate from the long axis. A protractor was then used to measure the angle formed and this value was recorded for each specimen.

Teeth with Type I, II, III, IV, and C-shaped mesial canal configurations (3) were allowed in our study. The canal configuration was determined when the initial file(s) were placed approximately 1 mm from the radiographic apex of the root. A Type I configuration was determined when only one canal was present in the mesial root. A type II configuration was identified when two canals were present in the mesial root that converged apically. A type III canal configuration existed when two canals were present in the mesial root and the canals remained separate to the apex. A Type IV canal system was determined when one canal in the mesial root split into two separate canals apically (2). A C-shaped canal may run the whole length of the root like a curtain or ribbon and exit at or near the root apex as a single foramen, or it may divide within the depth of the canal into two or more canals that exit separately (2).

PCR-BASED IDENTIFICATION OF *E. FAECALIS*

Detection of *E. faecalis* was performed using 16S rDNA polymerase chain reaction (PCR). The PCR protocol was adapted from Leys et al. (258). PCR analysis was used to confirm the presence of *E. faecalis* prior to instrumentation and to compare the ability of hand and rotary instrumentation and hand and rotary plus ultrasonic irrigation to eliminate *E. faecalis* from the mesial root canals of necrotic, infected lower molar teeth. Only agar plates demonstrating viable bacterial colonies prior to instrumentation (S1) and after hand and rotary instrumentation (S2) for teeth in Group 1 or after hand and rotary instrumentation (S2) and hand and rotary plus ultrasonic irrigation (S3) for teeth in Group 2 were chosen for PCR analysis. PCR was used to evaluate the presence of *E. faecalis* on eight agar plates representing four teeth from subjects in Group 1 and on ten agar plates representing five teeth from subjects in Group 2.

Reference DNA from *E. faecalis* strain ATCC 29212 (American Type Culture Collection, Manassas, VA) was extracted to serve as a positive control for the PCR reactions. For extraction of the positive control DNA, 1 mL of cultured strain ATCC 29212 was centrifuged at 10,000 x g for 30 seconds, after which the supernatant was removed and discarded. The pellet was re-suspended in 300 µL of 10% sodium dodecyl sulfate (SDS) in TE (pH 8.0), 10 µL of 3% proteinase K was added, and the sample was vortexed. Following an overnight incubation at 37°C, the DNA was purified by the standard GeneClean® (Bio 101, Inc., LaJolla, CA) protocol, with an additional wash step, and eluted in 20 µL of 10 mM Tris-HCl (pH 8.0).

Colony samples, which served as the DNA template for the PCR reactions, were obtained from an agar plate by touching the surface of each colony with a sterile micropipette tip. The pipette tips were subsequently transferred into 50 μ L of a PCR reaction mixture containing 10X PCR buffer (Amersham, Piscataway, NJ), 0.2 mM of each deoxynucleoside triphosphate (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate; Amersham, Piscataway, NJ), 3 mM $MgCl_2$, 2.5 U of *Taq* polymerase (Fisher Scientific, Pittsburgh, PA), and 0.0003 μ g/ μ L of primers ENFE (*E. faecalis* specific: 5'-GTC GCT AGA CCG CGA GGT CAT GCA-3', Biosynthesis, Lewisville, TX) and L189 (universal: 5'-GGT ACT TAG ATG TTT CAG TTC-3', Biosynthesis, Lewisville, TX). These primers produced an amplicon size of approximately 718 bp. The PCR mix was pipetted up and down to introduce the colony samples into the mix. PCR reactions containing ATCC strain 29212 and water served as positive and negative controls, respectively. The PCR amplification was performed in an automated thermal cycler (Perkin-Elmer Cetus, Wellesley, MA). The cycling parameters were: 27 cycles of [1 minute at 94°C (denaturation), 2 minutes at 52°C (annealing), and 3 minutes at 72°C (elongation)].

The PCR product was observed after electrophoresis of 10 μ L of the PCR reaction mixture on 1% agarose gel in TBE (0.1 M Tris-borate [pH 8.3], 2 mM EDTA). The gel was stained for 15 minutes with 0.5 μ g/mL ethidium bromide, de-stained with sterile water, and visualized under ultraviolet light. A positive or negative score for the presence of *E. faecalis* was assigned based on the presence of clear bands of the expected molecular size (approximately 718 bp). *Eco*RI and *Hind*III digestion products of

bacteriophage lambda DNA served as the molecular size standard. The presence or absence of *E. faecalis* in each sample was recorded.

A $\log_{10}$ transformation of each CFU count was performed to normalize the data from S1 and S2 samples prior to statistical evaluation due to the high range of bacterial cell numbers. A dependent t-test was used to detect significance in the reduction of bacteria from the initial sample (S1) to the post-instrumentation (S2) sample in each group. Between groups differences for continuous variables (age, size of periapical lesion, canal curvature, working length, initial CFU count, post-instrumentation CFU count, and post-ultrasonic CFU count) were analyzed using the Exact Mann-Whitney-Wilcoxon test. Between groups differences for dichotomous variables (gender, tooth type, canal type, final taper, S2 positive cultures, and S3 positive cultures) were analyzed using the Chi-Square test. Adjusted odds ratios for the presence of bacteria after treatment were determined for tooth type, canal type, instrument taper, and method of instrumentation using logistic regression.

CHAPTER 4

RESULTS

A total of 33 patients were used in our study. Group 1 (hand and rotary instrumentation with no ultrasound) consisted of 17 patients (51.5%), and Group 2 (hand and rotary instrumentation with ultrasound) consisted of 16 patients (48.5%). Table 1 shows a breakdown of the two experimental groups into canal curvature, canal type, tooth type, and size of pre-operative periapical radiolucency. A more detailed record of data (gender, age, tooth number, instrumentation method, presence of *E. faecalis*, working length, depth of ultrasonic needle penetration) is found in the Raw Data (Appendix H).

A summary for between group differences for continuous and dichotomous pre-operative variables is presented in Tables 2 and 3. Statistical analysis for these variables was completed on 31 subjects (16 from Group 1 and 15 from Group 2) since one outlying canal type was present in each group. Reliability was not determined for the main operator since each measurement was only taken once within individual subjects. Table 2 summarizes between group differences for continuous variables, including age, periapical lesion area, canal curvature, and working length. No statistically significant differences were found between Group 1 and Group 2 with regard to subject age ($p = 0.268$), size of periapical lesion ($p = 0.527$), canal curvature ($p = 0.406$), and working length ($p = 0.926$).

Between group differences for initial CFU count (S1), post-instrumentation CFU count (S2), and post-simple irrigation (Group 1) or post-ultrasonic irrigation (Group 2) CFU counts (S3) are found in Table 3. Statistical analysis for these variables was completed on 31 subjects (16 from Group 1 and 15 from Group 2) since one outlying canal type was present in each group. The mean $\log_{10}$ CFU count in the initial samples (S1) for the hand file and rotary instrumentation group with no ultrasonics (Group 1) was 5.30 ± 1.1 , with a range between 2.38 and 6.74. This corresponds to a CFU count of 1.10×10^6 , with a range between 2.40×10^2 and 5.44×10^6 (Tables 3 and 4, Figures 16 and 18). The mean $\log_{10}$ CFU count following hand file and rotary instrumentation with conventional syringe irrigation (S2) for Group 1 was 1.60 ± 1.70 , with a range between 0 and 5.26. This corresponds to a CFU count of 1.21×10^4 , with a range between 0 and 1.80×10^5 (Tables 3 and 4, Figures 16 and 19).

The mean $\log_{10}$ CFU count in the initial samples for the hand file and rotary instrumentation group with ultrasonics (Group 2) was 5.50 ± 0.8 , with a range between 4.45 and 6.61. This corresponds to a CFU count of 1.07×10^6 , with a range between 2.84×10^4 and 4.08×10^6 (Tables 3 and 4, Figures 17 and 18). The mean $\log_{10}$ CFU count following hand and rotary instrumentation with conventional syringe irrigation (S2) for Group 2 was 2.30 ± 1.8 , with a range between 0 and 4.94. This corresponds to a CFU count of 1.07×10^4 , with a range between 0 and 8.80×10^5 (Tables 3 and 4, Figures 17 and 19). The mean CFU count in the post-ultrasonic irrigation samples (S3) for the hand file and rotary instrumentation group with ultrasonics (Group 2) was 146.7, with a range between 0 and 1.52×10^3 (Table 3, Figures 17 and 20).

No statistically significant differences were found between Group 1 and Group 2 with regard to initial (S1) CFU count ($p = 0.385$) and post-instrumentation (S2) CFU count ($p = 0.093$). A trend toward significant reduction of CFU counts at S3 between groups was observed following ultrasonic irrigation, although the difference was not statistically significant ($p = 0.068$). The median, minimum, and maximum CFU counts by method of instrumentation are found in Table 3. Initial, post-instrumentation, and post-ultrasonic bacterial cell counts for each experimental sample are listed in Appendix H (Raw Data). Representative photographs of blood agar plates used for quantification of colony-forming units from each group are presented in Figures 4 through 10.

Table 4 summarizes the mean $\log_{10}$ bacterial cell counts and standard deviations pre- and post-instrumentation for Group 1 and Group 2. Statistical analysis for intra-group differences was completed on all 33 subjects (17 from Group 1 and 16 from Group 2). Group 1 and Group 2 both showed consistent decreases in bacterial cell counts from initial CFU counts following hand and rotary instrumentation. Table 4 shows Group 1 and Group 2 significantly ($p < 0.0001$) reduced the number of bacteria from initial counts.

Table 5 summarizes between group differences for dichotomous variables, including gender, tooth type, canal type, and final canal taper. Statistical analysis for these variables was completed on 31 subjects (16 from Group 1 and 15 from Group 2) since one outlying canal type was present in each group. No significant differences were found between Group 1 and Group 2 with regard to subject gender ($p = 0.321$), tooth type ($p = 0.474$), canal type ($p = 0.853$), and final taper ($p = 0.594$).

Between group differences for positive cultures at S2 and S3 is presented in Table 6. Statistical analysis for these variables was completed on 31 subjects (16 from Group 1 and 15 from Group 2) since one outlying canal type was present in each group. No significant differences were found between Group 1 and Group 2 with regard to the presence of bacteria post-canal preparation ($p = 0.321$). However, a statistically significant ($p = 0.038$) higher proportion of teeth in Group 2 were negative for bacterial growth following instrumentation and ultrasonic irrigation than for teeth in Group 1 following instrumentation and syringe irrigation.

The number of samples displaying positive growth at S1, S2, and S3 for Group 1 and Group 2 is found in Table 7 and illustrated in Figure 21. All 31 (100%) samples used for statistical analysis were positive for initial growth. Nine out of 16 (56%) samples were positive for bacterial growth in Group 1 and 11 out of 15 (73%) samples were positive for bacterial growth in Group 2 following hand and rotary instrumentation. Three out of 3 (100%) samples in Group 1 were positive for bacterial growth following additional irrigation. This sampling was completed on a limited number of teeth in Group 1 to confirm the minimal effect of additional irrigation on reducing the bacterial count or improving the likelihood of achieving a negative culture. Three out of 15 (20%) samples were positive for bacterial growth in Group 2 following ultrasonic irrigation. Table 8 summarizes the logistic regression analysis (adjusted odds ratios) for bacterial presence after treatment. Tooth type (second molar versus first molar, $p = 0.135$), canal type (type III versus type II, $p = 0.551$), and instrument taper (0.06 versus 0.04, $p = 0.278$) were found not to be significant. Method of instrumentation (non-ultrasound versus ultrasound, $p = 0.0021$) was significant. The use of ultrasonic irrigation following

hand and rotary instrumentation was 6.98 times more likely to yield a negative culture than when ultrasonic irrigation was not utilized.

Colonies of *E. faecalis* were isolated from 3 out of 4 (75%) initial (S1) samples and 1 out of 4 (25%) post-instrumentation (S2) samples from subjects in Group 1, and on 4 out of 5 (80%) initial samples (S1), 4 out of 5 (80%) post-instrumentation samples (S2), and on 1 out of 2 (50%) post-ultrasonic irrigation samples (S3) from subjects in Group 2 (Table 9). Too few samples were present to evaluate the statistical significance of the results from the PCR analysis. Representative photographs of agarose gels used for PCR identification of *E. faecalis* are presented in Figures 11-15.

CHAPTER 5

DISCUSSION

Selected portions of the following Discussion section were adapted from previous theses by Gutarts et al. (62), Goodman et al. (260), Lev et al. (261), Haidet et al. (262), and Archer et al. (263) from the Department of Endodontics at The Ohio State University.

DISCUSSION OF MATERIALS AND METHODS

An in vivo design was chosen for our study for several reasons. McComb and Smith (264,265) attempted to correlate results from two studies that evaluated chemomechanical instrumentation. The methods and materials in each study were similar, except that one study was in vitro and the other in vivo. The authors concluded the in vitro design allowed for a more aggressive approach, which increased debridement efficacy as compared to in vivo instrumentation. Walton (215) and Bolanos and Jensen (266) also reported on differences in instrument manipulation that would increase the effectiveness of in vitro instrumentation. Fairbourn et al. (267) stated that positive apical pressures present in the mouth were difficult to simulate in vitro. The authors concluded this difference in apical pressure might alter canal preparation in an in vivo situation.

The intent of our study was to have the parameters of the experimental treatment conform as much as possible to a clinical setting. Therefore, an in vivo design was utilized.

Our study was limited to teeth with a clinical diagnosis of pulpal necrosis with acute or chronic periapical periodontitis and a periapical radiolucency on the mesial root of the tooth. Research has demonstrated the presence of viable bacteria in the root canals of necrotic teeth with periapical radiolucencies. Kakehashi, Stanley, and Fitzgerald (64) in a classic study involving germ-free gnotobiotic rats established the importance of bacteria in the etiology of apical periodontitis. Pulpal and periapical pathosis were only seen in the presence of bacteria. In a study involving teeth with necrotic pulps due to trauma, Wittgow and Sabiston (76) demonstrated that 32 of 40 intact teeth with necrotic pulps had an infected pulp and 97% of these harbored obligate anaerobes. Sundqvist (74) in a study of 32 necrotic anterior teeth due to trauma determined that bacteria could be isolated from necrotic pulps only when periapical destruction was evident. Sundqvist (74) further found that 90% of the organisms isolated from the necrotic teeth were anaerobic and emphasized the use of an anaerobic glove box for isolation and purification of root canal isolates. In a study analyzing the effectiveness of increased apical enlargement on bacterial reduction in root canals, Card et al. (31) found that 95% of the test teeth diagnosed with necrotic pulps and apical periodontitis showed the presence of viable bacteria in the root canal.

Conversely, past research also demonstrated that vital teeth diagnosed with irreversible pulpitis are essentially free of bacteria. Shuping et al. (30) in a study comparing the reduction of intracanal bacteria with nickel-titanium rotary instruments and various medications showed the absence of bacteria in 4 out of 5 teeth diagnosed

with irreversible pulpitis. Keudell et al. (268) also failed to demonstrate the presence of anaerobes in teeth with vital pulp chambers.

Therefore, since bacteria were frequently isolated from necrotic teeth with evidence of periapical destruction, only teeth with a clinical diagnosis of pulpal necrosis with acute or chronic periapical periodontitis and a minimum 2 x 2 mm mesial periapical radiolucency were used to test the ability of ultrasonic irrigation, following hand and rotary instrumentation, to further reduce the population of viable bacteria within infected root canals.

Mandibular molars were chosen to serve as experimental teeth because these teeth frequently require endodontic therapy (3). Mesial canals were chosen due to the complexity of their root canal anatomy (5-13). Skidmore and Bjorndal (9) found 60% of mandibular molar mesial roots, with two canals, had some form of transverse communication. This communication was most commonly observed in the apical third of the root. The authors noted that an operator attempting to remove the pulp tissue must be aware of these transverse communications. Pineda and Kuttler (5) examined radiographs of extracted mandibular molars with a magnifying glass. The authors reported ramifications (offshoots) of the main canal in the mesial root in 48% of first molars, 28% of second molars, and 19% of third molars. The authors also observed the majority of these ramifications were located in the apical third of the root. Cambruzzi and Marshall (211) reported that, in the mesial root of 108 mandibular molars, an isthmus joined two canals 60% of the time. Two mesial canals were found without an isthmus in only 18% of the cases. Vertucci (11) found that 63% of first molars and 31% of second molars had a transverse communication in the mesial root. In approximately 75% of first and second

molars this communication was found in the middle third of the root. Yesilsoy et al. (13) examined freshly extracted mandibular first and second molars after injecting vinyl polysiloxane impression material into the pulp chambers and carefully removing and measuring the impressions with a millimeter ruler. The ruler, at zero, placed at the mid-floor area of the impression and viewed from the mesial, measured (to the nearest 0.5 mm) the depth of the mesial groove between the mesiolingual and mesiobuccal canals. The average recorded depth was 1.0 mm. Some of the impressions had depths measuring 3.5 mm. This was a significant space when considering the limitations of root canal instrumentation techniques. The authors postulated this area may remain un-debrided after instrumentation thereby affecting the prognosis of treatment in both vital and necrotic teeth. The authors believed that modifications in access preparation and/or an increased emphasis on irrigation and intracanal medication may be needed. Hess (10) concluded that apical ramifications are often observed in the roots of upper molars and the mesial roots of lower first and second molars. The author reported a fine connecting canal or fissure was found between the two main canals in the mesial roots of lower molars.

Gutarts et al. (62) studied the effects of an ultrasonic irrigating needle as an adjunct to hand and rotary instrumentation in the mesial root canals of 36 mandibular molars with a clinical diagnosis of irreversible pulpitis. The authors found significantly ($p < 0.05$) less dentinal debris and pulp tissue remaining following the use of the ultrasonic irrigating needle regardless of canal type, canal curvature, or apical level. Canal isthmuses were also significantly cleaner ($p < 0.05$) following the use of the ultrasonic irrigating needle. The authors concluded the use of the ultrasonic irrigating

needle with 6.0% NaOCl for 1 minute is clinically applicable and better able to debride the root canal system than hand and rotary instrumentation alone. Card et al. (31) discussed the importance of communications between the mesial canals of mandibular molars in a study analyzing the effect of increased apical enlargement on the reduction of intracanal bacteria. Following instrumentation to an apical size ProFile® #7, 93% of sampled canals without a clinically detectable communication cultured bacteria-free. In contrast, only 64% of sampled canals with a clinically detectable communication cultured bacteria-free. The authors stated that even with crude criteria 41% of instrumented mesial canals were found to communicate, and further stated this percentage was most likely higher. In addition, a likely problem with studies by Dalton et al. (29) and Shuping et al. (30) on the reduction of intracanal bacteria following instrumentation with nickel-titanium rotary instruments and saline irrigation (29) and instrumentation with nickel-titanium rotary instruments and the placement of calcium hydroxide as an inter-appointment dressing (30) was the mesiolingual canal was not instrumented, which may have increased positive results due to the joining of the mesiobuccal canal with the mesiolingual canal. These results emphasize the importance of eliminating bacteria from the approximate 65% of mandibular molars that have a direct communication between the mesiobuccal and mesiolingual canals (128).

Therefore, the high percentage of fins, isthmuses, and accessory canals found in mesial roots of mandibular molars and the reported difficulty in cleaning these areas served as an excellent testing ground to determine if ultrasonics provided greater bacterial reduction in canals after hand and rotary instrumentation.

The irrigating solution used in our study was 6.0% sodium hypochlorite (“Ultra”). A 6.0% sodium hypochlorite concentration was used instead of a 5.25% concentration (“Regular”) because the 5.25% solution is no longer being marketed. The 6.0% solution was not diluted to a 5.25% concentration because that would not be clinically applicable. In view of the fact we were trying to duplicate clinical therapy situations, 6.0% sodium hypochlorite was utilized instead of 5.25% sodium hypochlorite.

In prior in vitro studies on the antibacterial effectiveness of endodontic irrigants, Harrison and Hand (202) found 5.25% sodium hypochlorite was the most effective antibacterial agent. These authors determined dilution of the irrigant significantly reduced the antibacterial efficacy of sodium hypochlorite. Shih et al. (197) also determined that full strength sodium hypochlorite was sufficient to sterilize artificially infected root canals while diluted sodium hypochlorite was not sufficient. Other in vivo studies have used diluted concentrations of sodium hypochlorite. Byström and Sundqvist (124) determined that 0.5% sodium hypochlorite was effective in eliminating bacteria from 12 of 15 necrotic root canals over a period of five appointments. In a study of the effectiveness of increased apical enlargement in reducing intracanal bacteria, Card et al. (31) yielded an 88.9% reduction in intracanal bacteria following irrigation with 1% sodium hypochlorite and apical instrumentation sizes in excess of 0.575 mm. Matsumiya and Kitamura (269) showed the number of bacteria decreased when canals were instrumented to larger sizes, and it is likely the increased size of instrumentation in the Card et al. (31) study had an equal effect in reducing intracanal bacteria as the chosen irrigant. Shuping et al. (30) found a 71.4% reduction of intracanal bacteria when canals were irrigated with 1.25% sodium hypochlorite and instrumented to sizes equal to those

in the Card et al. (31) study. None of the studies using a diluted concentration of sodium hypochlorite were able to predictably render canals bacteria-free.

Harrison et al. (270) conducted a clinical study to determine the effect of various endodontic irrigants on inter-appointment pain. The authors found no difference in the incidence of inter-appointment pain between 5.25% sodium hypochlorite and physiologic saline. Yguel-Henry et al. (271) showed that sodium hypochlorite increased the cutting efficacy of files (+200% for the K-file and +30% for the H-file) due to its lubricating properties. No study has compared the antibacterial efficacy of 5.25% sodium hypochlorite to 6.0% sodium hypochlorite, although empirically 6.0% NaOCl should be more effective at reducing intracanal bacteria. Therefore, based on these studies, 6.0% sodium hypochlorite was used in our study due to its antimicrobial effectiveness and clinical applicability.

A 25-gauge, 5/8-inch, Luer-Lok hypodermic needle was chosen for non-ultrasonic irrigation for several reasons. Authors such as Abou-Rass and Piccinino (272) and Chow (273) felt irrigation effectiveness was a function of placement depth of the irrigating needle. Therefore, the authors recommended the use of a 27- or 30-gauge needle. However, Archer et al. (27) found the lumen of a 30-gauge needle clogged immediately when used with 5.25% sodium hypochlorite. A 27-gauge needle clogged after only a few minutes of use when used with the same solution. A 25-gauge needle was able to express the solution freely during the entire period of instrumentation. The choice of a 25-gauge needle conforms to the recommendation of Harrison (274), who advocated the use of a 25- to 27-gauge needle. The 5/8-inch length was chosen since it could easily be manipulated in the pulp chamber. This length was also chosen because it

was more clinically applicable than using the same 25-gauge, 1.5-inch needle for both the ultrasonic and non-ultrasonic groups. The needles were bent at the junction of the hub and needle at approximately 45 degrees. This bend allowed the needle to be easily placed in the canal to a point just short of binding. This point was estimated to be approximately 15 to 17 mm from the coronal aspect of the access opening, and corresponded to the middle third of the canal. The 5/8-inch needle was inserted to this point in the canal. Therefore, a longer needle was not required.

During instrumentation, 2 mL of irrigating solution were deposited in each canal after the use of every third hand and rotary file, after the orifice openers, and after the #5 Gates-Glidden drill were used in the root canal. This amount was used in previous studies (15-17,27) and it follows the recommendation of Harrison (274), who recommended 2 to 5 mL of 5.25% sodium hypochlorite after every instrument. This recommendation was based on the trapping of air bubbles in the canal system that prevented contact of the irrigant with soft tissue attached to the canal. The mechanical movement of instruments disrupted the air bubbles and allowed the irrigant to move throughout the system. We irrigated after every third hand and rotary file, after the orifice openers, and after the #5 Gates-Glidden drill instead of irrigating after every instrument because this was more clinically applicable. This type of protocol still allowed for the disruption of any air bubbles and provided ample sodium hypochlorite in the canal system to help remove pulpal and dentinal debris.

Although uncommon in modern endodontics, bacterial sampling of root canals was once routine practice. As late as 1985, Trope and Grossman (275) reported that 51% of dental schools taught culturing while 37% of undergraduate and 20% of postgraduate

programs cultured clinically. The tendency to culture has declined with the advent of single-visit endodontics; however, culturing is still valuable, especially in cases of non-healing periapical lesions such as those associated with *Actinomyces* infections (276).

The field decontamination protocols outlined by Möller (177) for microbial sampling of root canals are generally agreed upon to be the most effective method for culturing of root canals and the periapical tissues with paper points. Möller (177) recommended cleaning the operative field with 30% hydrogen peroxide followed by disinfection of the tooth and rubber dam with 5% or 10% iodine tincture. Using this technique, Möller (177) achieved 83.3% to 98.2% decontamination on teeth with or without restorations. Ng et al. (178) compared the use of 10% iodine tincture and 2.5% sodium hypochlorite for field decontamination prior to bacterial sampling for contemporary microbiologic techniques and achieved 81% decontamination with iodine and 72% decontamination with sodium hypochlorite on teeth without restorations. The author also achieved 81% decontamination on Oraseal placed at the junction between the tooth and rubber dam with the use of iodine tincture. The author concluded that 2.5% sodium hypochlorite may be a more effective field disinfectant for contemporary techniques; however, no difference was observed between iodine tincture and sodium hypochlorite for traditional culture methods.

The field decontamination protocols of Möller (177) were used in our study with few exceptions. After the patient was anesthetized and the rubber dam was in place, the tooth, adjacent rubber dam, and rubber dam clamp were cleansed with 30% hydrogen peroxide until no further effervescence of the peroxide occurred. The peroxide was vigorously scrubbed on all surfaces for a period of 30 seconds using sterile cotton swabs.

If bubbling was incessant, Cavit[®] was placed around the neck of the tooth and the process repeated. This ensured saliva could not penetrate the field of operation. The tooth, rubber dam, rubber dam clamp, and Cavit[®] were then disinfected with Povidone-Iodine 10% Swabsticks. Povidone-iodine 10% was used due to the potentially caustic nature of 5% iodine tincture, its rapid antiseptic action against a wide range of microorganisms, low toxicity, and hypoallergenicity (277). The Swabsticks were used for the convenience of application. The povidone-iodine was allowed to dry for 2 minutes.

The access opening was made with sterile #4 round burs, the canals located, and sterile orifice shaper rotary files (ProFile[®] GT[®] size #70/.12, #50/.12, and #35/.12) were utilized for coronal flaring of the distal canal. Another set of identical sterile orifice openers were then utilized for coronal flaring of the mesial canals, without lubricant, followed by 2 mL of sterile 0.9% sodium chloride irrigation of the pulp chamber only. Sterile orifice openers were used prior to bacterial sampling in order to facilitate the placement of the paper points used for the sampling procedure as close to the estimated working length as possible. In addition, by using these instruments there was less coronal binding and better direct access to the apical third of the canal. Therefore, the instruments were less likely to separate and the rotary files could more easily work at the apex. Also, a reservoir was formed for the sodium hypochlorite and lubricating solutions. Toward the end of instrumentation the ProFile[®] GT[®] #70/.12, #50/.12, and #35/.12 orifice shaper rotary files were again used to further enlarge the coronal aspect of the canal to allow the 25-gauge ultrasonic needle to penetrate the canal as deep as possible and move freely without binding.

Cavit[®] was used to occlude the distal canal during instrumentation of the mesiobuccal and mesiolingual canals and during bacterial sampling of the mesiobuccal and mesiolingual canals to prevent cross-contamination of bacteria from the distal canals into the mesial canals. Two mL of sterile 0.9% sodium chloride irrigation were again used to flush any debris from the pulp chamber. Using the pre-operative radiograph, a preliminary working length was determined by making an approximate measurement 0.5 mm short of the radiographic apex. The pulp chamber was then dried with sterile cotton pellets, and 0.02 mL of liquid dental transport fluid (LDT, Anaerobe Systems, Morgan Hill, CA) were placed in the mesiobuccal and mesiolingual canals with a sterile tuberculin syringe. This same amount of LDT was used in previous studies (30,31) and is small enough to restrict the transport medium to the root canal and prevent overflow into the pulp chamber. LDT is a reduced transport fluid consisting of buffered salts with sodium thioglycollate and cysteine added to provide a reduced environment. This medium is advocated for use in the collection and transportation of anaerobic bacterial dental specimens (278). Past studies (72-77) indicated that 90% or greater of bacteria isolated from necrotic root canals are anaerobic. Griffie et al. (172), in an in vivo study of 17 necrotic teeth, compared trypticase soy broth and reduced thioglycollate media and found oxygen must be removed from the transport fluid to retrieve anaerobic isolates. Carlsson and Sundqvist (175), Zielke et al. (171), and Goodman (279) also suggested the use of reduced transport media when culturing necrotic root canals. Therefore, reduced transport media was used to recover the predominantly anaerobic isolates within infected root canals.

The canal contents were dispersed into the LDT with sterile #10-20 K-files placed to within 1 mm of the estimated working length and agitated in a coronal-to-apical motion. Sterile x-fine or fine paper points were then placed in the mesiobuccal and mesiolingual canals, as close as possible to the working length, and then transferred to a vial containing 1.0 mL of LDT. Paper point transfer of microorganisms was used based on the work of Shuping et al. (30), Card et al. (31), and Marshall and Savoie (174). Using similar methods for paper point transfer, Shuping et al. (30) and Card et al. (31) recovered bacteria from 99% of pre-instrumentation samples. Marshall and Savoie (174) compared in vitro wet and dry paper points for the collection of bacteria from infected root canals. Paper points moistened with saline yielded growth in 93% of samples, whereas dry paper points yielded growth in only 36% of samples. Therefore, based on these studies, the initial (S1) and all subsequent bacterial samples in our study were taken with sterile, moist, paper points.

Following collection of the initial sample, hand and rotary instrumentation was used to initiate debridement of the mesial canals. Initially, smaller size #10-20 K-files were used to enlarge the canals to a size #20 K-file. This was done to remove the bulk of pulp tissue, determine working length, and prevent any potential canal blockage that may occur if a larger sized #30 rotary file was used initially. These smaller files also gave the operator the ability to explore the canal anatomy by using tactile sense that would be difficult to do using rotary instrumentation. The Root ZX[®] Apex Locator along with a #10 K-file was used to determine the actual working length for each root canal instrumented in the study. The unit was set for 0.5 mm from the apex. After electronic apex location, a radiograph was exposed with a slight mesial angulation (approximately

15° from perpendicular) to determine the position of each #10 K-file, and to confirm the working length determined by the Root ZX[®] Apex Locator. This radiograph also helped determine canal configuration. The use of a working length 0.5 mm short of the apex was used due to the work of Kuttler (7). The author showed the greatest canal constriction was located in dentin, 0.507-0.784 mm coronal to the apical cementum. Green (8) reported this canal constriction to be an average of 0.75 mm from the apical opening. The author further stated the apical opening could be at the true apex of the tooth, or any location up to 2 mm eccentric to this position.

The Root ZX[®] Apex Locator was used to find the working length because it was shown accurate at finding the working length of a canal (280-283) when a potential error of (+/-) 0.5 mm from the foramen was used. The Root ZX[®] was reported to locate the foramen with a clinical accuracy rate of 96% as reported by Shabahang et al. (280), 100% as found by Pagavino et al. (281), and 83% as reported by Meares et al. (282). El Ayouti et al. (283) showed that radiographic working length determination resulted in overestimation in 51% of root canals tested, although the measuring file tip was assessed to be 0 to 2 mm short of the radiographic apex. Electronic working length measurements with the Root ZX[®] were more accurate than radiographic film since the apex locator overestimated the working length in only 21% of the canals tested. Additional studies using other apex locators have shown that working lengths determined by electronic apex locators were significantly more accurate than working lengths determined by radiographs (284,285). Brunton et al. (284) showed the Analytic A.F.A. electronic apex locator was extremely accurate in locating the apical foramen when 25 (100%) teeth were tested within 0.5 mm of the anatomical apex and 11 (44%) teeth were tested at the apical

foramen. In contrast, 15 (60%) teeth tested using radiographs alone were within 0.5 mm of the anatomical apex and only 4 (16%) teeth were actually at the anatomical apex. Pratten et al. (285) determined the working length of root canals in human cadaver teeth by positioning an endodontic file at the apical termination point as indicated by the Endex Apex Locator. Radiographs were then taken at various angles with the file in place. Five examiners determined a radiographic termination point for each canal evaluated on the radiographs. The teeth were extracted and examined histologically to determine the ideal termination point. The deviations of the two experimental termination points from the ideal termination point were compared. The mean of the absolute value of the deviations from the apical constriction for the apex locator was significantly less ($p < 0.05$) than the mean for the radiographic method. Therefore, the method using the apex locator was considered more reliable. Consequently, the Root ZX[®] Apex Locator was used in our study to determine working length, and a radiograph was used to confirm this length and determine the canal type.

Nickel-titanium rotary files, used in a crown-down method, were chosen as the primary method of instrumentation because these files were shown to be an effective method of reducing intracanal bacteria in vivo (29-31). Dalton et al. (29) compared the ability of 0.04 tapered nickel-titanium rotary instruments and K-flex files in a step-back instrumentation sequence to reduce intracanal bacteria in 48 patients with apical periodontitis. Sterile saline was used for irrigation. The authors demonstrated complete elimination of bacteria in only 28% of samples with no significant differences between the two instrumentation regimens. The authors stated the high percentage of positive cultures in the molar samples may be due to communications between the mesiobuccal

canal and the un-instrumented mesiolingual canal. The authors concluded the addition of antimicrobial irrigants such as sodium hypochlorite to the instrumentation regimen is important in reducing intracanal bacteria. Shuping et al. (30) evaluated the extent of bacterial reduction with nickel-titanium rotary instrumentation and 1.25% sodium hypochlorite irrigation. The additional antibacterial effect of placing calcium hydroxide for greater than 1 week was also tested. The authors found a significantly greater pattern of bacterial reduction when sodium hypochlorite was used as an irrigant, compared with the results of the Dalton et al. (29) study, in which sterile saline was used as the irrigant ($p < 0.05$). After instrumentation with sodium hypochlorite irrigation, 61.9% of canals were rendered bacteria-free. The placement of calcium hydroxide for at least 1 week rendered 92.5% of the canals bacteria-free. This was a significant reduction compared with sodium hypochlorite irrigation alone ($p = 0.0001$). The results of this study indicated that sodium hypochlorite irrigation with rotary instrumentation is an important step in the reduction of canal bacteria during endodontic treatment. However, this method could not consistently render canals bacteria-free. Card et al. (31) studied the effectiveness of increased apical enlargement in reducing intracanal bacteria in teeth with a clinical and radiographic diagnosis of apical periodontitis. Two mandibular canines, 11 premolars, and 2 molars were instrumented using 0.04 taper ProFile® rotary files and 1% sodium hypochlorite irrigation. The canines and premolars were instrumented to a #8 size and the molars to a #7 size. Final instrumentation was performed with LightSpeed® files and 1% sodium hypochlorite irrigation to a size #60 for molars and a size #80 for canines and premolars. The authors demonstrated 100% bacterial reduction in the canine and premolar samples and 81.5% reduction in the molar samples after the first

instrumentation. Following the second instrumentation, 89% of the molar samples were bacteria-free. In molar samples without a clinically detectable communication, 93% were bacteria-free after the first instrumentation.

Nickel-titanium rotary files, used in a crown-down technique, were also shown to maintain the curvature of the root canal, and produce uniform and round preparations (23,24,26). Producing uniform preparations conducive to adequate obturation of the root canal space was necessary in our study, since the teeth used were not later extracted as in the Gutarts et al. (62) study. Hulsmann et al. (23) evaluated the straightening of curved root canals, post-operative root canal diameter, safety issues (file fractures, perforations, apical blockages, loss of working length), cleaning ability, and working time using two different rotary nickel-titanium instruments: Hero 642[®] and Quantec[®] SC. Both Ni-Ti systems maintained curvature well. Most procedural incidents occurred with Quantec[®] SC instruments: 5/25 (20%) instruments were fractured, 3/25 (12%) had apical blockages, and 8/25 (32%) cases lost working length. Preparation with Hero 642[®] instruments resulted in 12% of canals with blockages and 4% with perforations. Following preparation with Hero 642[®], 63% of the root canals showed a round, 24% an oval, and 17% an irregular cross-sectional shape. Quantec[®] SC preparations resulted in a round shape in 24% of the cases, oval shape in 29%, and an irregular cross-section in 47% of the cases. SEM was used to investigate cleanliness of the root canal walls using a five-score system for debris and smear layer. In terms of debris, the Hero 642[®] system achieved better results than the Quantec[®] SC system. The results for smear layer were similar: cleaner root canal walls were found after preparation with Hero 642[®] than with Quantec[®] SC. Both systems maintained original root canal curvature well and showed

good cleaning ability. Tan and Messer (26) compared the quality of apical enlargement of mesiobuccal canals of mandibular molars using conventional stainless steel hand files (K-files) and nickel-titanium (Ni-Ti) rotary instruments (LightSpeed®). Canal cleanliness, canal transportation, and final canal shapes were determined histologically at 1 mm and 3 mm levels short of the working length. Canals were prepared to significantly larger sizes using LightSpeed® instrumentation than with hand instrumentation (15-17 ISO units, $p < 0.001$). LightSpeed® instrumentation allowed greater apical enlargement with significantly cleaner canals, less apical transportation, and better canal shape than both hand instrumentation groups at both levels ($p < 0.05$). None of the three techniques were totally effective in cleaning the apical canal space.

The ProFile® GT® rotary file system (Dentsply Tulsa Dental, Tulsa, OK) was chosen for the bulk of canal preparation in our study because it is a widely used brand throughout the United States. These nickel-titanium rotary files have little risk of apically transporting, ledging, or perforating the tooth (286). Variable pitched flutes provide reamer-like efficiency at the shank and K-file strength at the tip, virtually eliminating the chance of accidental breakage. The ProFile® GT® rotary instruments pre-defined taper lets the operator create a continuous tapering preparation with just a few instruments (286). A crown-down method of instrumentation was used with the ProFile® GT® rotary files. This method of instrumentation allowed larger tapered rotary files to open and flare the canals coronally and let each subsequently smaller tapered rotary file to descend apically into the canal in order to debride and shape the apical aspect. Coldero et al. (130) compared in vitro intracanal bacterial reduction using nickel-titanium rotary instruments with and without apical enlargement. Thirty-eight palatal roots of

maxillary molar teeth were inoculated with *E. faecalis* and prepared in a step-down sequence with engine-driven ProFile® GT® rotary files at 350 rpm with or without additional apical enlargement. The authors found no significant difference in intracanal bacterial reduction when ProFile® GT® rotary preparation with 4.4% NaOCl and 15% EDTA irrigation was used with or without additional apical enlargement, suggesting it may not be necessary to remove dentin in the apical part of the root canal when a suitable coronal taper is achieved to allow satisfactory irrigation of the root canal system with antimicrobial agents. Rollison et al. (131) and Siqueira et al. (132) also compared reduction of intracanal bacteria utilizing several instrumentation techniques including GT® files with or without additional apical enlargement. Rollison et al. (131) compared the in vitro antibacterial effectiveness of two nickel-titanium rotary instrumentation systems differing in sequence and apical enlargement size. Forty-four mesiobuccal canals of mandibular molars inoculated with radioactively labeled *E. faecalis* were instrumented either to an apical size #35 with GT® and ProFile® rotary files or to an apical size #50 with Pow-R® rotary instruments. Phosphate-buffered saline was used for irrigation. The authors found that instrumentation to an apical size #50 with Pow-R® rotary instruments significantly reduced intracanal bacteria better than GT® or ProFile® rotary files ($p < 0.05$). Siqueira et al. (132) evaluated the in vitro ability of hand Nitiflex files, GT® files, and ProFile® 0.06 Tapers™ Series 29™ to reduce intracanal populations of *E. faecalis*. Sterile saline was used for irrigation. Instrumentation to a Nitiflex #40 file significantly reduced more intracanal bacteria than any other technique tested. The authors concluded these instrumentation techniques, along with sterile saline irrigation, effectively eliminate greater than 90% of intracanal bacteria. Additional studies (22,287)

assessed the ability of the ProFile® GT® instrumentation system to debride the root canal. Schafer and Zapke (22) showed that rotary ProFile® instruments (crown-down technique) had the best debridement results, especially in curved canals, when compared to hand instrumentation performed with K-Flexofiles used in a reaming motion and a step-back technique, Hedström files used in a filing motion, and mechanical rotary preparation using K-Flexofiles. Iqbal et al. (287) reported that rotary ProFile® instrumentation or ProFile® GT® pre-instrumentation had no effect on canal transportation and loss of working length regardless if used in a crown-down or step-back instrumentation method.

Following hand and rotary instrumentation of the mesial canals in our study, a second bacterial sample (S2) was collected for teeth in both groups. This sample was collected using the pumping maximum removal method originally described by Möller (177), with the exception that LDT was used as the transport medium instead of VGMA II. LDT has the clinical advantage of being a liquid instead of a gel and still maintains the viability of bacteria for 72 hours (278). Möller compared six different sampling techniques in 489 teeth of 373 patients. These techniques included: (1) a file was used in a moist or dry canal to scrape dentin filings from the canal wall; (2) a moist paper point was wiped along the canal wall in a moist or dry canal; (3) dry paper points were used in a fluid filled canal without prior pumping motion from a file for partial and (4) maximum removal of fluid in the canal; (5) dry paper points were used in a fluid filled canal with prior pumping motion using a file for partial and (6) maximum removal of fluid in the canal (PMR, pumping maximum removal, method). The PMR method was accomplished with the coarsest file that could be inserted into the entire length of the prepared part of the root canal and pumped vigorously for 1 to 2 minutes. In root canals prepared 1 to 2

mm from the apex with variable bone rarefaction, the PMR method yielded growth in 41% of samples as compared to 13% with technique 1, 16% with technique 2, 18% with technique 3, 31% with technique 4, and 33% with technique 5. The PMR method used with sampling fluid (VGMA II) in the canal yielded significantly ($p < 0.001$) more samples with positive growth than any of the other techniques. In our study, after instrumentation, each mesial canal was dried with sterile medium and coarse paper points, flushed with 2 mL of 5% sodium thiosulfate for 1 minute to neutralize the sodium hypochlorite (177), and rinsed with 2 mL of sterile 0.9% sodium chloride irrigation. The canals were dried with sterile medium and coarse paper points and 0.02 mL of LDT were placed in the mesiobuccal and mesiolingual canals using a sterile tuberculin syringe. A sterile K-file equal in size to the master apical file was placed to working length and pumped five times with minimal reaming motion to disperse the canal contents into the LDT. Sterile medium and coarse paper points were then used to absorb the canal contents and the paper points were transferred to a vial containing 1.0 mL of LDT.

Ultrasound was added in our study after completion of hand and rotary instrumentation in Group 2 to enhance antimicrobial efficacy. A number of studies that have used ultrasound after hand instrumentation have demonstrated greater bacterial reduction than hand instrumentation alone. Sjögren and Sundqvist (55) analyzed the antibacterial effect of ultrasonic root canal instrumentation in 31 human, single-rooted necrotic teeth with periapical radiolucencies. Instrumentation was performed with a Cavi-Endo® ultrasonic unit using 0.5% sodium hypochlorite as an irrigant. Bacterial samples were taken prior to and after instrumentation, after ultrasonic instrumentation, and at each successive appointment. The authors determined that ultrasonic activation

eliminated bacteria from canals more effectively than hand instrumentation alone, and concluded the use of an antibacterial dressing is necessary to achieve as complete a reduction in bacterial numbers as possible. Spoleti et al. (57) evaluated the in vitro effect of passive ultrasonic activation on root canal disinfection. An inoculum consisting of *S. aureus*, *S. viridans*, and *E. coli* was placed in each canal. Passive ultrasonic activation was performed with 0.5 mL of sterile saline and a #20 K-file attached to a Cavi-Endo® ultrasonic unit for 10 seconds. The number of surviving colonies was higher when ultrasonics was not used. The authors concluded ultrasonic activation is beneficial in disinfection of the root canal system, but is difficult to use in roots of small sizes and complete disinfection of the canal cannot be achieved without antiseptic irrigants. Huque et al. (59) evaluated ultrasonic intracanal irrigation procedures in eradicating bacteria from surface, shallow, and deep layers of root dentin using extracted human teeth. Ultrasonic irrigation with 5.5% and 12% sodium hypochlorite eradicated bacteria from artificial smear layer ($p < 0.0001$), while 12% sodium hypochlorite irrigation with a syringe was insufficient. Ultrasonic irrigation with less concentrated sodium hypochlorite failed to eliminate bacteria completely from reservoir channels in most samples. Ultrasonic irrigation with 12% sodium hypochlorite appeared to eliminate bacteria efficiently from surface, shallow, and deep layers of root dentin.

Ultrasonic instrumentation in the absence of sodium hypochlorite was reported by Ahmad et al. (58) in an in vitro study and Alaçam et al. (288) in an in vivo study to have little beneficial effect in reducing the population of viable bacteria within the root canal. Cunningham et al. (217), in a study comparing the antimicrobial effectiveness of ultrasonic with hand instrumentation using either saline or sodium hypochlorite as an

irrigant, determined the presence of sodium hypochlorite was important to achieve bacterial reduction with either ultrasonic or hand instrumentation. Ahmad et al. (45-47) and Walmsley (49) stated that ultrasonic instrumentation was more effective in canal debridement after hand instrumentation as compared to ultrasonic instrumentation alone, because the vibrating ultrasonic tip was able to freely translate without dampening. Ultrasonic file dampening was thought to cause a loss of energy when the file contacted the root canal wall. Hand and rotary preparation also allowed for a greater volume of irrigating solution to be circulated in the root canal space, thereby increasing debridement efficacy (274,289-290). Krell et al. (291) examined irrigation patterns during ultrasonic canal instrumentation in clear resin blocks. A Cavi-Endo[®] unit was utilized with Endosonic files. Using a food color dye, the authors found the irrigant could not reach the most apical extent of the preparation until the ultrasonic file could vibrate freely within the canal. Ahmad et al. (45) reported a significant difference in smear layer removal when ultrasound was used with either sodium hypochlorite or water. Cameron (252) also reported on the synergistic relationship between ultrasound instrumentation and sodium hypochlorite, stating that ultrasound with 2% or 4% sodium hypochlorite was able to remove the smear layer from extracted teeth.

Jahde et al. (292) established the safe in vivo use of ultrasound in a study that compared short-term periapical responses to hand and ultrasonic file overextension during root canal instrumentation in monkeys. Using either 2.6% sodium hypochlorite or saline as an irrigating solution, #25 files were used 1 mm past the radiographic apex. A Cavi-Endo[®] unit was used in the ultrasonic part of the study. At 48 hours post-

instrumentation, no significant difference was found in inflammatory reaction among any of the groups. All inflammatory reactions were of low to moderate intensity.

The MiniEndo™ ultrasonic endodontic system was used in our study for several reasons. Since this device is a piezoelectric unit, it generates minimal heat and does not require an external water coolant supply. The ultrasonic energy is developed when a high frequency alternating current is applied to a piezoelectric material, such as barium titanate or lead zirconate titanate. Corresponding lengthening and shortening of the material then occurs over a minute distance, which produces ultrasonic vibrations that are transferred to the tip of the instrument (50). The MiniEndo™ operates by the action of cavitation following the propagation of ultrasonic energy in a frequency spectrum between 25 and 32 kHz and amplitudes ranging from 5 to 250 µm at more than 20,000 cycles per second. A computer chip is found in the base of the MiniEndo™, which reads the ultrasonic amplitude at the tip. The computer checks this amplitude 7 times a second. The computer chip regulates the amplitude at the tip to a constant rate by boosting power to the handpiece, even when resistance is encountered. This feedback mechanism and the constant tip amplitude separate the MiniEndo™ from other piezoelectric units (293).

The Enac unit, similar to the MiniEndo™, is a quartz piezoelectric unit that vibrates at 30,000 cycles per second. The Enac can be used in endodontic and periodontal procedures to deliver ultrasonic energy and a continuous flow of irrigation from the head of the handpiece. The irrigant does not pass through the ultrasonic tip. Instead, the irrigant is sprayed from the head of the handpiece and flows along the side of the ultrasonic tip or file. The Enac unit has wider amplitude at the tip than the MiniEndo™ that may lead to fracture of small ultrasonic tips necessary in endodontic

procedures (294). Ahmad (140), using an Enac unit, was unable to produce cavitation at lower power settings and effectively eliminate *Bacteroides intermedius* from active bacterial cultures.

The Cavi-Endo[®] unit utilizes a magnetostrictive generator that must have a water coolant source due to the production of excessive heat in the handpiece. This requirement for a coolant source necessitates that a special water coolant attachment be plumbed into the dental unit. The MiniEndo[™] system was also reported to be more efficient than the Cavi-Endo[®] system because the electromechanical conversion efficiency of magnetostrictive units is only about 50%, compared to 70-80% for piezoelectric units (50). Jensen et al. (19) reported a canal cleanliness value of 83.3% for the canal surface running from the canal apex to the 6 mm level when the MiniEndo[™] unit was used at low power for 3 minutes with a #15 file.

A 25-gauge, 1.5-inch needle was chosen for use in the ultrasonic phase of our study since it was shown to produce the most cavitation and acoustic streaming in a previous pilot study by Gutarts et al. (62) when compared to 27- and 30-gauge, 1.5-inch needles. Each needle was operated in the MiniEndo[™] ultrasonic device (Figure 1) at a maximum power of 10 and observed with a Jedmed microscope (6.0x-magnification). The 30-gauge needle showed the least amount of acoustic streaming (confirmation of nodes and antinodes) followed by the 27-gauge needle. The highest amount of acoustic streaming was observed objectively when the 25-gauge needle was used. The 25-gauge needle was the only needle that was able to produce cavitation (confirmation of bubble mist). This same pilot study also showed that when the 25-gauge needle tip was blunted, it produced no cavitation and very little acoustic streaming. The 1.5-inch length was

chosen because it permitted clearance for high-volume evacuation and the longer length provided the ability to see inside the access chamber during ultrasonic instrumentation because the needle was exposed 20 mm past the hub. An ultrasonic needle was chosen instead of an ultrasonic file because the needle could deliver sodium hypochlorite directly into the root canal, which enabled the solution to be delivered continuously to the apex and also replenish the canal with new solution (Figure 2).

The use of ultrasonics during irrigation procedures may lead to an increase in external root surface temperature that may result in damage to the PDL. Cameron (295) reported the use of ultrasonics with an intermittent flow of irrigant led to a temperature peak of 45°C internally and 40°C externally, whereas continuous flow caused external temperature to fall from 37°C to 32°C. The rise in temperature following intermittent irrigation may be significant, since Eriksson and Albrektsson (296) showed that heat-induced bone necrosis occurs at 10°C above normal body temperature of 37°C sustained for 1 minute. Sodium hypochlorite was delivered continuously in our study through the irrigation needle, which may help to cool the root and prevent damage to the PDL. A third bacterial sample (S3) was taken for teeth in Group 2 following ultrasonic irrigation in an identical manner to the collection of S2.

A power setting of 10 (maximum power) was used when the MiniEndo™ was activated because Gutarts et al. (62) in a pilot study demonstrated the 25-gauge needle could run at full power without breaking for more than 1 minute. The combination of a 25-gauge needle and a full power setting was able to produce excellent cavitation and acoustic streaming.

Previous studies using ultrasonic instrumentation demonstrated that longer time periods at lower power settings were needed in order to not break the ultrasonic files while still achieving good tissue debridement (14-21,27). Ahmad et al. (47) determined that cavitation could be produced at a minimum power setting of 3.5 (out of 10), and that a minimum displacement amplitude of 135 microns was required for the file to oscillate freely (without binding) in a canal prepared apically to a #40 file. Archer et al. (263) conducted a pilot study using a #15 Endosonic file in an Enac ultrasonic unit to determine if higher power settings could be used clinically. The authors found using ultrasonically instrumented acrylic blocks that at a power setting above 5.0 files fractured within three minutes. At a setting of 5.0, severe apical transportation of the canal (zipping) occurred. At a power setting of 4.0, mild to moderate apical zipping occurred in the blocks after three minutes. No apical zipping was observed after three minutes at a power setting of 3.5. Zipping was not a concern in our study since the needle was never placed within the apical third of a tooth due to its thickness. The needle was used in the middle to coronal third where it could vibrate freely without wall contact.

The decision to use ultrasound for 1 minute in each canal was based on the desire to reduce working time in each canal while increasing the ultrasonic energy delivered. Lev et al. (16) showed increased efficacy in isthmus cleaning at the 1 mm and 3 mm apical levels when a Cavi-Endo[®] ultrasonic unit and a #20 ultrasonic file were used for 3 minutes as opposed to 1 minute. We wanted to show that a three-minute time period is not needed if the ultrasonic instrument (in our study, a needle) can be used at the higher energy produced by the MiniEndo[™] unit. Therefore, we used a more clinically applicable time period of 1 minute.

Continuous irrigation (15 mL) delivered 6.0% sodium hypochlorite at a rate of 15 mL/minute through intravenous tubing connected directly to the ultrasonic needle. This type of system was chosen since it can easily deliver the solution at a continuous and steady rate without interruption during intracanal irrigation. Previous studies (14-18,33-44,46,50,51,244,251,294) also used continuous irrigation in conjunction with ultrasonic instrumentation but reported problems in the in vivo setting because the irrigating solution was applied using a reservoir in the ultrasonic unit (14,17,33-44,46,50,51,244,251,294) or an intravenous bag (15,16,18). If an intravenous bag were used, a special hookup would be required to suspend the bag. Also, a constant flow rate would not be achieved; especially at the rate we wanted (15 mL/minute), unless the bag was used in conjunction with a pump. In our study we used a syringe operated by a human to deliver the irrigant at a rate of 15 mL/minute. The use of a diffusion pump would probably control the flow rate more precisely. After using sodium hypochlorite as an irrigating solution, the reservoir in the Cavi-Endo[®] unit requires flushing due to mineral deposits and the corrosive action of sodium hypochlorite on metal (293). Also, the reservoir is small and would need to be replenished with irrigating solution after irrigating each canal. This would delay the operator and increase working time. When continuous irrigation is used in the Enac unit, water must be used as an irrigant because the unit will get too hot and there is a risk of possible degradation of the irrigating solution. In addition, the irrigant is sprayed out of the handpiece and streams down the file. The irrigant is never delivered directly into the canal where it has a better chance of reaching the apex and disinfecting the root canal. Problems were also found when intermittent irrigation was used with ultrasonic instrumentation (211). Ultrasonic

instrumentation had to be stopped every 30 seconds in order to deliver more irrigating solution due to quick depletion of the irrigant from the pulp chamber.

Gutarts et al. (62) indicated that trying to deliver sodium hypochlorite at a rate of 20 mL/minute initially resulted in pain due to possible pressure and extrusion of the sodium hypochlorite into the periapical tissues. The rate was slowed down to 15 mL/minute. Our study also delivered sodium hypochlorite at a rate of 15 mL/minute. After the sodium hypochlorite was fully delivered, a total of 15 mL in each canal was dispensed using ultrasonic irrigation over a one-minute time period. Gutarts et al. (62) showed that patients had no pain during ultrasonic irrigation when 6.0% sodium hypochlorite was delivered at a rate of 15 mL/minute, whereas mild to moderate pain was experienced when a rate of 20 mL/minute was used after debridement with hand and rotary instrumentation.

Previous studies comparing hand instrumentation to a hand instrumentation plus ultrasound technique have added extra irrigating solution, like our study, to the instrumentation group to control for the amount of irrigating solution used in the experimental groups. Goodman et al. (15) added an additional 3 minutes of continuous 40 mL/minute/canal of 2.62% sodium hypochlorite irrigation, either with or without ultrasonic activation. Lev et al. (16) added additional instrumentation with a #20 file and continuous flow of 30 mL/minute/canal of 2.62% sodium hypochlorite irrigating solution to control the variables in all groups. Haidet et al. (17) also used additional filing with a #20 file and 30 mL/minute/canal additional irrigation (5.25% sodium hypochlorite) to control for filing time and the volume of irrigating solution in the experimental groups. Therefore, in our study, following collection of S2, three subjects in Group 1 received an

additional 15 mL of 6.0% sodium hypochlorite for 1 minute in each mesial canal, as a control procedure. Although this may seem clinically inapplicable, the additional irrigation in the instrumented/non-ultrasonic group was done in order to control for the instrumentation variables except for the experimental factor (ultrasonication). A third bacterial sample (S3) was taken for these three subjects in an identical manner to collection of S2. This controlled the effect additional irrigation only may have on bacterial reduction by having both groups irrigated the same way.

Random assignment to experimental groups was made after completion of the hand and rotary instrumentation. Therefore, canal curvature and difficulty in instrumentation did not influence preparation of the canals. Fifteen envelopes per experimental group were initially placed into a box and shuffled. Due to the unpredictability of available patients for this in vivo study, we did not know how many experimental teeth could be treated. The minimum number of teeth in each experimental group was set at fifteen. Therefore, after the initial 30 envelopes were used, each successive experimental treatment was decided by a draw between a pair of envelopes, containing a representative from each experimental group. A parallel control group was used in this study and consisted of teeth with mesial canals instrumented with a hand and rotary technique only. A parallel control group was used in order to blind the investigator to the treatment provided. The absence of a control group may have inadvertently affected the way that each canal was instrumented, since the type of instrumentation would be known prior to initiation of treatment.

Root curvature was also considered to be an important factor in our study since it may have an effect on potential bacterial reduction. In a study evaluating the efficacy of

root canal preparations, Bolanos and Jensen (266) found that with greater root curvature, round preparations were more difficult to achieve. Walton (215) found that fewer canal walls were planed in canal preparation when the root curvature increased. Using radiographs exposed from a buccal direction, Schneider's (259) method was used in our study to determine root curvature. Previous studies (15-17,27) also used Schneider's method to determine root curvature. Canals with a curvature of 25 degrees or less were designated as moderately curved, and canals with a curvature of 26 degrees or greater were designated as severely curved. These degrees of curvature were chosen in order to have the same amount of specimens in the two separate curvature groups. These classifications closely resemble Schneider's classifications of moderately curved (less than 25 degrees), and severely curved (25 to 70 degrees) canals (259).

Root configurations, as described by Weine (3), were also evaluated in our study. Canal type was initially determined when the working length radiograph was exposed from a mesial angulation. In mesial roots with a Type I canal configuration, only 1 canal was present. A Type II canal configuration existed when two separate canals joined to form a common canal at the apex of the root. In teeth with a Type II canal configuration, filing alternated between buccal and lingual canals to avoid blockage of the canals at the point of intersection. Type III canals remained separate throughout the length of the canal. A type IV canal system splits into two separate canals apically, which may complicate instrumentation of both canals (3). One canal may not be instrumented because the operator is unaware of the apical canal bifurcation or is unable to gain access to the canal due to its severe divergence from the main canal. A C-shaped canal can also be difficult to instrument since the ribbon shaped canal may run the whole length of the

root and exit at or near the root apex as a single foramen, or it may divide within the depth of the canal into two or more canals which exit separately (2).

Each bacterial sample was transported in LDT immediately to a dental microbiology laboratory at The Ohio State University for quantification of colony forming units. Although LDT will sustain microorganisms for up to 72 hours (278), the samples were transported and prepared immediately in order to minimize any loss of viable bacteria. A negative control for unwanted bacterial growth during the microbiologic preparation of samples was created by placing sterile paper points in LDT, making serial dilutions of the control sample, and plating these dilutions on blood agar plates. Any demonstrable growth on blood agar arising from the negative control samples indicated contamination, and any growth arising from associated experimental samples was dismissed. Each bacterial sample was vortexed for 30 seconds to disperse the contents within the LDT vial and to dislodge any bacteria that may be attached to the enclosed paper points.

Ten-, 100-, and 1000-fold serial dilutions of the original sample were prepared in the following manner: (1) the original sample was vortexed for 30 seconds; (2) 30 μ L of the original sample were transferred aseptically with a micropipette to 270 μ L of LDT to create a 1/10 dilution; (3) 30 μ L of the 1/10 dilution were transferred aseptically with a micropipette to 270 μ L of LDT to create a 1/100 dilution; and (4) 30 μ L of the 1/100 dilution were transferred aseptically with a micropipette to 270 μ L of LDT to create a 1/1000 dilution. The sample was diluted due to the possibility of large numbers of viable bacteria in the original sample, which may produce a lawn of bacteria on the agar surface and make quantification of distinct colonies difficult if not impossible. The maximum

number of countable colonies on an individual agar plate was considered to be 250 (148). Therefore, a 1/1000-dilution plate could sustain 10^7 organisms. Since Akpata (297) reported the average number of microorganisms in an infected tooth to be 10^6 , further dilution was not necessary. A total of 300 μL of sample fluid was present in each microcentrifuge tube. This volume was used to provide a margin of error since 250 μL was the amount used to inoculate each agar plate.

Each experimental and control sample was streaked on an anaerobic sheep blood agar plate (Brucella blood agar, Anaerobe Systems, Morgan Hill, CA). This enrichment medium is formed by the addition of citrated blood to tryptic soy agar, which permits differentiation of some species of bacteria based on characteristic hemolytic patterns and will support the growth of many fastidious organisms commonly isolated from cerebrospinal fluid, pleural fluid, sputum, and wound abscesses (148). Anaerobic sheep blood agar plates were used for cultivation of bacterial samples in our study based on the work of Shuping et al. (30) and Waltimo et al. (161). Shuping et al. (30) used anaerobic sheep blood agar to cultivate organisms isolated from infected root canals following nickel-titanium rotary instrumentation with 1.25% NaOCl irrigation and medication with calcium hydroxide. The anaerobic sheep blood agar yielded growth on 98% of initial specimens, 62% of specimens following nickel-titanium rotary instrumentation, and in 7.5% of specimens following medication with calcium hydroxide. Waltimo et al. (161) used Brucella blood agar to compare in vitro the susceptibility of oral *Candida* species and *E. faecalis* to calcium hydroxide solutions at a pH of 12.4 for time periods ranging from 5 minutes to 6 hours. Colony-forming units were counted after 24 hours and 48

hours of incubation. Growth on the agar plates indicated equal resistance to calcium hydroxide for the strains of oral *Candida* and *E. faecalis* used in this study.

A streak plate technique was used in our study for isolation of individual colonies of bacteria. The microbial mixture (250 μ L) was transferred to the center of an anaerobic sheep blood agar plate utilizing a micropipette with sterile pipette tips and then streaked over the surface with a sterile 60 mm cell spreader (Fisher Scientific, Pittsburgh, PA) while spinning the agar plate in a clockwise manner on a plate-spinner. The plate-spinner permitted consistent rotation of the agar plates, which assisted even distribution of the sample on the surface of the agar. Aliquots of 250 μ L of undiluted and diluted specimens were used based on the work of Shuping et al. (30). Using this technique, single cells dropped from the cell spreader as it was streaked along the agar surface and developed into separate colonies. The agar plates were then incubated at 37°C for 7 days in an anaerobic glove box containing 5% carbon dioxide, 10% hydrogen, and 85% nitrogen. Wittgow and Sabiston (76) isolated microorganisms from intact teeth with necrotic pulps and determined that 97% of these teeth yielded obligate anaerobes. Sundqvist (74) found that 90% of organisms isolated from necrotic teeth were anaerobic. Therefore, all sample incubation of agar plates in our study took place under anaerobic conditions. Zielke et al. (171) in a comparison of non-rereduced PRS and TSB determined that incubation for 7 days is required for adequate retrieval of bacterial isolates. Sundqvist (74) also determined that anaerobic plates should be incubated for 7 days or more to permit the development of distinguishable colony characteristics. Therefore, all agar plates in our study were incubated for a period of 7 days.

Individual colony forming units were counted following incubation using a microscope at 10x magnification. Only agar plates with colony forming units numbering less than 250 were counted, since separation of distinct colonies was not possible on plates with more than 250 colony-forming units. The number of colony-forming units per sample was calculated using the formula: $((\#CFU \times 4) \times 10^{|y|+1} \times 1 \text{ mL LDT/per sample vial}) = \#CFU/\text{sample}$, where y = dilution factor used in a specimen (30). For an undiluted specimen, $y = |0| = 0$, for a 10-fold (1/10) dilution, $y = |-1| = 1$, for a 100-fold (1/100) dilution, $y = |-2| = 2$, and for a 1000-fold (1/1000) dilution, $y = |-3| = 3$.

The presence of *E. faecalis* was also considered important in our study since *E. faecalis* is associated with endodontic treatment failure (102,103,143,160-163).

Detection of *E. faecalis* was performed using 16S rDNA polymerase chain reaction (PCR). Past studies have used PCR for the detection of *E. faecalis* within infected root canals. Rôças et al. (187) used PCR to detect the presence of *E. faecalis* in infections of endodontic origin and to determine if this species is associated with different forms of periradicular disease. The authors detected *E. faecalis* in 18% of clinical specimens from teeth with primary apical periodontitis and 67% of clinical specimens from previously root-filled teeth. Rôças et al. (188) used 16S rDNA-based PCR to identify the occurrence of 9 endodontic pathogens in previously root-filled teeth in a South Korean population. *Enterococcus faecalis* was the most frequently detected species, found in 64% of isolates. Baumgartner et al. (189) used PCR to detect geographical differences of specific species of bacteria including *E. faecalis* in abscesses of endodontic origin obtained from Portland, OR and Rio de Janeiro, Brazil. The authors isolated *E. faecalis* from 16% of specimens from Portland and 17% of samples from Rio de Janeiro. The authors

concluded the use of primers highly specific for the organisms tested allows for precise detection of these organisms by PCR. Siqueira et al. (157) used PCR and checkerboard DNA-DNA hybridization to detect the prevalence of *Actinomyces* species, streptococci, and *E. faecalis* in 53 infected teeth. The polymerase chain reaction used with ubiquitous primers detected the presence of bacteria in all 53 samples. Checkerboard DNA-DNA hybridization detected the presence of *Actinomyces* species in 9.4% of samples, streptococci in 23% of samples, and *E. faecalis* in 7.5% of samples.

Only agar plates demonstrating viable bacterial colonies prior to instrumentation and after hand and rotary instrumentation for teeth in Group 1 or after hand and rotary instrumentation and hand and rotary plus ultrasonic irrigation for teeth in Group 2 were chosen for PCR analysis. Distinct colonies demonstrating growth on agar plates represent individual bacterial species (148). Therefore, samples from all viable colonies on the agar plate used for quantification of colony forming units were taken for use in the PCR analysis. For example, if 20 colonies were present on a 1/1000-dilution agar plate of a pre-instrumentation sample, then a sample from all 20 colonies was obtained for use in the PCR analysis.

The procedure for isolation and purification of DNA, the specific PCR protocol, and all DNA extraction and PCR reagents were adapted from Leys et al. (258). Using this PCR protocol, Leys et al. (258) detected *A. actinomycetemcomitans* in 60% and *P. gingivalis* in 79% of saliva and plaque samples obtained from 52 subjects. The 16S gene-coding region of the prokaryotic genome contains sequences unique to a single species (258). Therefore, oligonucleotide primers specific for the 16S ribosomal operon of *E. faecalis* were used to obtain rDNA spacer region fragments specific for *E. faecalis*. The

use of these highly specific spacer region fragments prevented cross-reactivity with even closely related species, enhancing the species specificity of the PCR assay. The ENFE forward primer sequence used binds at position 1266 bp of the 16S ribosomal operon of the *E. faecalis* genome. The total length of the 16S ribosomal operon is 1522 bp. Therefore, the length of the PCR amplicon size from its binding position on the 16S ribosomal operon to the spacer region is 256 bp. The L189 reverse primer sequence used binds at position 211 bp of the 23S ribosomal operon of *E. faecalis*. Therefore, the length of the PCR amplicon size from its binding position on the 23S ribosomal operon to the spacer region is 211 bp. The length of the spacer region was 251 bp. Therefore, the total PCR amplicon size was expected to be 718 bp (Figure 3). The use of *Eco*RI and *Hind*III digestion products of bacteriophage lambda DNA as the molecular size standard was also based on the work of Leys et al. (258). This marker produces bands of known molecular size during electrophoresis, which were used to determine the size of amplicons on the agarose gel. Reference DNA extracted from *E. faecalis* strain ATCC 29212 was used as a positive control in order to reduce the possibility of false-positive results. Past studies (187,188) have used this strain as a positive control for the detection of *E. faecalis* from infected root canals by PCR. A positive or negative score for the presence of *E. faecalis* was assigned based on the presence of clear bands of the expected molecular size (718 bp).

A log₁₀ transformation of each CFU count was performed to normalize the data from S1 and S2 samples prior to statistical evaluation due to the high range of bacterial cell numbers. A dependent t-test was used to detect significance in the reduction of bacteria from the initial sample (S1) to the post-instrumentation (S2) sample in each

group. This test was used since the same subjects were measured on a numerical variable before and after instrumentation. Between groups differences for continuous variables (age, size of periapical lesion, canal curvature, working length, initial CFU count, post-instrumentation CFU count, and post-ultrasonic CFU count) were analyzed using the Exact Mann-Whitney-Wilcoxon test. This test was utilized to evaluate the independent variables because of skewed distributions; so parametric testing was not possible. This non-parametric test indicated which factors may have had a significant effect on the reduction of intracanal bacteria. This test also provided exact p-values instead of approximate values obtained from a statistical table. Between groups differences for dichotomous variables (gender, tooth type, canal type, final taper, S2 positive cultures, and S3 positive cultures) were analyzed using the Chi-Square test. This test was used to compare frequencies among the dichotomous variables, and to determine if bacterial presence was associated to the method of instrumentation. Adjusted odds ratios for the presence of bacteria after treatment were determined for tooth type, canal type, instrument taper, and method of instrumentation using logistic regression. This test determined the utility of the ultrasonic technique in the presence of potential confounders. Logistic regression allows analysis of odds ratios when the independent variables include both numerical and nominal measures and the outcome variable is binary (dichotomous). In our study, the outcome variable was defined as yes or no for the presence of bacteria after treatment.

DISCUSSION OF RESULTS

Sample distributions for both groups, in terms of canal curvature, canal anatomy, tooth type, and size of periapical radiolucency is found in Table 1. A total of 17 mandibular molars were used in the no ultrasound group (Group 1), and 16 mandibular molars were used in the ultrasound group (Group 2). Statistical analysis on between group differences for continuous and dichotomous variables was completed on 31 subjects (16 from Group 1 and 15 from Group 2) since one outlying canal type was present in each group. This equal distribution of samples in both groups allowed for a comparison analysis to determine if one technique was superior to the other based on the experimental factor (ultrasound), and eliminating the possible influences of unequal sample size. Equal distribution of sample size was also utilized in previous studies where bacterial reduction was compared using hand or ultrasonic instrumentation/irrigation (60,139,141,288). Siqueira et al. (60) utilized an equal sample size distribution comprised of 4 groups of 20 teeth each. Group 1 received manual irrigation with 4% NaOCl, group 2 received ultrasonic irrigation with 4% NaOCl, group 3 received manual alternate irrigation with 4% NaOCl and 3% hydrogen peroxide, and group 4 received manual irrigation with sterile saline. DeNunzio et al. (139) utilized two experimental groups with 24 samples receiving ultrasound instrumentation and 20 samples receiving step-back hand instrumentation. Fegan and Steiman (141) had eight groups of 10 teeth each. Groups 1 and 2 were control groups, group 3 received hand instrumentation with sterile water, group 4 received hand instrumentation with 5.25% NaOCl, group 5 received ultrasonic irrigation with sterile water, group 6 received ultrasonic irrigation with 5.25% NaOCl, group 7 received laser irradiation with sterile water, and group 8

received laser irradiation with 5.25% NaOCl. Alaçam et al. (288) had two equal groups of 50 teeth each. Fifty teeth were treated with hand instrumentation and alternate irrigation with 5% NaOCl and 3% hydrogen peroxide and 50 teeth were treated with ultrasonic irrigation with 5% NaOCl.

In order to have the most statistical power and statistical efficiency between the two experimental groups in our study, it was important to have an equal and somewhat large sample size for each experimental group. A large sample size may better represent information about each group, while a small sample size may give skewed or non-representative information about the same group. It was also important to have similar population sizes in each experimental group in order to avoid confounding variables such as canal type, canal curvature, tooth (root) length, instrument taper, and tooth type. These confounding variables may influence the results of the experiment even though each has no impact on the experimental variable (ultrasound). In an ideal situation, it is best to stratify each experimental group with equal populations. However, since this is extremely difficult to do in a clinical, in vivo experiment, it was important to be aware of results potentially impacted by confounding variables. Therefore, in order to obtain an accurate evaluation of the experiment it was important to have sample sizes in the experimental groups as equal as possible.

In our study, canal curvature was separated according to Schneider (259) into moderately curved (0-25°) and severely curved (> 25°) groups. Schneider's method (259) was used to determine canal curvature. Forty-seven percent of the mesial roots in Group 1 and 44% of Group 2 canals were considered severely curved (Table 1). Mean canal curvature was not significantly different ($p = 0.4057$) between Group 1 and Group 2 in

our study (Table 2). The mean canal curvature for Group 1 was 20.4°, and the mean canal curvature for Group 2 was 23.1°. Since the mean canal curvature was very similar and not significantly different between Group 1 and Group 2, statistical analysis was not performed to determine if ultrasonic irrigation was more effective in reducing the intracanal population of bacteria in roots of varying curvature.

No studies analyzing the antibacterial effectiveness of instrumentation or ultrasonic irrigation procedures have included canal curvature as an independent variable. Instead, the majority of studies (57,59,60,130-135,140-142) have used single-canal teeth with presumably straight roots for in vitro experiments. Coldero et al. (130) used root sections obtained from the palatal roots of maxillary molars to determine intracanal bacterial reduction with and without apical enlargement. Canal curvature was not recorded; however, the authors did not use any canal with a curvature greater than 5° as determined by Schneider's method. Pataky et al. (135) used extracted human maxillary first premolars to determine the antimicrobial efficacy of different root canal preparation techniques. The authors stated maxillary first premolars were specifically used since severe root curvatures are rare in this group of teeth. Shuping et al. (30) did not record canal curvature; however, the authors did instrument straight canals to a larger apical size with rotary instruments, which may have impacted bacterial reduction.

In our study, each canal of each tooth was instrumented to a .04 or .06 taper and an apical size of 0.30 mm using ProFile® GT® rotary instruments and K-type hand files. Nine (29%) samples in Group 1 and 7 (23%) samples in Group 2 out of 31 total teeth submitted for statistical analysis were instrumented to a .04 taper, and 7 (23%) samples in Group 1 and 8 (26%) samples in Group 2 out of 31 total teeth submitted for statistical

analysis were instrumented to a .06 taper (Table 5). The determining factor was canal curvature and size with regard to final instrument taper. Forcing a larger tapered instrument into narrow or excessively curved canals may result in separation of the instrument, and it was our goal to avoid this circumstance if possible. No instruments were separated during the course of our study. No significant difference ($p = 0.594$) was found between groups with regard to instrument taper (Table 5). Increasing taper of the canal preparation did not improve the likelihood of obtaining a negative culture (Table 8).

The inclusion parameters of our study allowed for mesial roots with canal types I, II, III, IV, and C-shaped as described by Weine (3). Canal type distribution by group is found in Table 1 and Table 5. Table 1 and Table 5 show an equal distribution of canal types between the two groups in our study. Eight (26%) samples in Group 1 and 8 (26%) samples in Group 2 out of 31 total teeth submitted for statistical analysis demonstrated a Type II canal system, and 8 (26%) samples in Group 1 and 7 (23%) samples in Group 2 out of 31 total teeth submitted for statistical analysis demonstrated a Type III canal system (Table 5). No significant differences ($p = 0.853$) were found between groups with regard to the type of canal system (Table 5). Therefore, the type of canal system did not have an impact on the reduction of intracanal bacteria between Group 1 and Group 2. Canal type also did not affect the likelihood of achieving a negative culture (Table 8).

Since we did not have any Type IV canals and only one C-shaped canal in our study, further studies are needed with the ultrasonic instrument used in our study on these types of complex canal systems. This may be difficult to do since these canal types are difficult to find in any appreciable number. Pineda and Kuttler (5) reported a 7.6% occurrence of Type IV mesial root configurations in a study of 300 mandibular second

molars, and Vertucci (11) reported a 9.0% occurrence of Type IV mesial root configurations in a study of 100 mandibular second molars. Sidow et al. (309) reported a 2.2% occurrence of C-shaped root configurations in a study of 150 mandibular third molars, while Weine (310) reported a 7.6% occurrence of C-shaped root configurations in a study of 811 mandibular second molars. C-shaped root forms are reportedly common in Asian populations (319). A study targeting subjects of Asian descent may increase the likelihood of obtaining C-shaped canals. One tooth with a Type I configuration was encountered in our study and included in Group 2. Since only one Type I and one C-shaped canal were included in our study, statistical analysis regarding the impact of canal type did not include this data. Canal type was analyzed for 16 subjects in Group 1 and 15 subjects in Group 2, all with either a Type II or III canal system. In order to avoid these confounding variables, these two subjects were also not included in the statistical analysis regarding between group differences for continuous and dichotomous variables.

Distribution of tooth types in each group is found in Table 1 and Table 5. Eleven (36%) samples in Group 1 and 12 (39%) samples in Group 2 out of 31 total teeth submitted for statistical analysis were from first molars, and 5 (16%) samples in Group 1 and 3 (10%) samples in Group 2 out of 31 total teeth submitted for statistical analysis were from second molars (Table 5). No significant difference ($p = 0.474$) in tooth type was found between Group 1 and Group 2 (Table 5). Tooth type may be an important factor in an in vivo study since access to the canals may impact the ability of the provider to properly instrument the canals. Impeded access may also limit penetration of an irrigating needle into the root canal and impair the removal of bacteria from the apical extent of the canal. Baumgartner and Falkler (73) demonstrated the predominance of

anaerobic bacteria in the apical 5 mm of infected human root canals. Therefore, limited access to the canals may impede the removal of these bacteria if inadequate instrumentation and irrigation is performed. The use of an ultrasonic irrigating needle may overcome these limitations.

The mean periapical radiolucency size for samples in Group 1 was 34 mm² and for Group 2 was 36 mm² (Table 2). No significant difference ($p = 0.527$) was found between Group 1 and Group 2 when comparing periapical radiolucency size (Table 2). Our study further confirmed the relationship between the presence of bacteria in the root canal and the development of periapical lesions. All subjects in our study presented with radiographic signs of apical periodontitis, and 100% of subjects contained cultivable bacteria in the initial sample. This is a similar finding to other studies (29-31,129,136,298) that cultured teeth with radiographic signs of apical periodontitis. Dalton et al. (29) cultured bacteria from 46 of 48 (96%) subjects with radiographic evidence of apical periodontitis. Shuping et al. (30) recovered bacteria from 41 of 42 (98%) test subjects with clinical and radiographic signs of apical periodontitis. Card et al. (31) retrieved bacteria from 38 of 40 (95%) subjects with radiographic evidence of apical periodontitis. McGurkin-Smith et al. (298) recovered bacteria from 29 of 31 (94%) subjects with radiographic evidence of a periapical lesion. Ørstavik et al. (129) demonstrated the presence of bacteria in 22 of 23 (96%) teeth with a radiographic diagnosis of apical periodontitis. Peters et al. (136) found microorganisms in 42 of 42 (100%) initial samples taken from the root canals of teeth with periapical radiolucencies.

VIABLE BACTERIA RECOVERY RESULTS

Initial Sampling (S1) of Bacteria

The mean $\log_{10}$ CFU count in the initial samples for the hand file and rotary instrumentation group with no ultrasonics (Group 1) was 5.30 ± 1.1 , with a range between 2.38 and 6.74. This corresponds to a CFU count of 1.10×10^6 , with a range between 2.40×10^2 and 5.44×10^6 (Tables 3 and 4, Figures 16 and 18). The mean $\log_{10}$ CFU count in the initial samples (S1) for the hand file and rotary instrumentation group with ultrasonics (Group 2) was 5.50 ± 0.8 , with a range between 4.45 and 6.61. This corresponds to a CFU count of 1.01×10^6 , with a range between 6.8×10^3 and 4.08×10^6 (Tables 3 and 4, Figures 17 and 18). No significant difference ($p = 0.385$) was found in the initial CFU counts for teeth in Group 1 and Group 2 (Table 3). Therefore, the reduction and any differences in post-instrumentation and post-ultrasonic CFU counts were not related to or dependent upon the initial number of intracanal bacteria. Our initial count was comparable to past studies (29-31,129,136,298) that analyzed the effect of instrumentation procedures on the reduction of intracanal bacteria. The mean $\log_{10}$ CFU count in the initial samples of the Dalton et al. (29) study was 4.60. In the Shuping et al. (30) study, the mean $\log_{10}$ CFU count in the initial samples was 5.51. The mean CFU count in the initial samples of the Card et al. (31) study was 6.0×10^7 , with a range between zero and greater than 10^9 . Ørstavik et al. (129) found CFU counts of 1×10^5 to 4×10^5 before the onset of treatment. The mean CFU count in the Peters et al. (136) study prior to the start of treatment was 1.0×10^6 , with a range between 80 and 3.0×10^7 . The mean bacterial count in the McGurkin-Smith et al. (298) study was 1.01×10^7 , with a range between 0 and greater than 10^9 . These counts are also comparable to the classic

research of Akpata (297), who determined the average number of bacteria in the infected dental pulp to be 10^6 . The similarity in initial counts between our study and the above-mentioned studies confirms that our methodology for obtaining and processing anaerobic specimens was valid. Representative photographs of agar plates with bacterial growth obtained from initial specimens in our study are found in Figures 4-7.

Bacterial Reduction Following Hand and Rotary Instrumentation (S2)

The mean $\log_{10}$ CFU count following hand file and rotary instrumentation with conventional irrigation (S2) for Group 1 was 1.60 ± 1.70 , with a range between 0 and 5.26. This corresponds to a CFU count of 1.17×10^4 , with a range between 0 and 1.80×10^5 (Tables 3 and 4, Figures 16 and 19). The mean $\log_{10}$ CFU count following hand and rotary instrumentation with conventional syringe irrigation (S2) for Group 2 was 2.30 ± 1.8 , with a range between 0 and 4.94. This corresponds to a CFU count of 1.07×10^4 , with a range between 0 and 8.80×10^5 (Tables 3 and 4, Figures 17 and 19). No significant difference ($p = 0.093$) was found in the post-hand and rotary instrumentation CFU counts for teeth in Group 1 and Group 2 (Table 3). Nine (29%) test teeth in Group 1 and eleven (35.5%) test teeth in Group 2 out of 31 total teeth submitted for statistical analysis showed positive cultures in the post-instrumentation sample (Table 6). In total, 64.5% of S2 samples from both Group 1 and Group 2 produced a positive culture. No significant differences ($p = 0.321$) were found in the percent of samples with positive bacterial growth at S2 between Group 1 and Group 2 (Table 6). Therefore, any further reduction in CFU counts beyond S2 was not related to or dependent upon the post-instrumentation number of intracanal bacteria.

Hand and rotary instrumentation with conventional irrigation resulted in a significant decrease ($p < 0.0001$) of intracanal bacteria from S1 to S2 in both Group 1 and Group 2 (Table 4). This significance was due to the large mean reduction in the number of intracanal bacteria between S1 and S2, and also the consistently low standard deviation values relative to the mean at S2. The low standard deviation seen in Groups 1 and 2 indicated low variance in bacterial reduction. This strongly indicates that hand and rotary instrumentation with intermittent 6.0% NaOCl irrigation consistently resulted in significant bacterial reduction from initial bacterial counts. Representative photographs of agar plates used for quantification of colony forming units at S2 are found in Figures 8 and 9.

In our study, hand and rotary instrumentation with conventional irrigation did not predictably render canals bacteria-free. Only 35.5% of post-instrumentation samples from both Group 1 and Group 2 produced a negative culture. The failure of nickel-titanium rotary instrumentation to consistently eliminate intracanal bacteria in our study may have resulted from tooth curvature, the anatomy of mesial roots, including canal type and the presence of transverse communications, and tooth type. The size of apical instrumentation, instrument taper, and the choice of irrigating solution may also influence bacterial reduction with hand and rotary instrumentation. Each of these areas will be discussed.

Canal curvature was reported to negatively affect canal cleanliness (23,26). The antimicrobial effectiveness of rotary instrumentation with nickel-titanium files may be affected by canal curvature. During rotary instrumentation with nickel-titanium files, all of the canal wall may not be instrumented because the curvature of the canal may cause

the files to contact certain portions of wall more than others (23,26). Since a nickel-titanium file is predisposed to remain straight, in apical areas it will touch the wall away from the curve (outer wall of a curved canal) and not touch the wall nearest to the curve (inner wall of a curved canal). Coronally, the file will contact the wall nearest to the apex of the curve (inner wall of a curved canal) and not touch the wall away from the curve (outer wall of a curved canal). Stainless steel hand files do not have the flexibility to bend around canals as well as nickel-titanium files and adapt even less to the anatomy of the canal (26). This leads to the same problems described above for nickel-titanium files, and may also result in the formation of ledges or zips in the canal that may further complicate instrumentation and leave areas of the canal not debrided. Pre-bent stainless steel hand files may help to overcome these limitations when instrumenting around curved canals. Cunningham and Senia (318), however, determined proximal curvatures (curves in a buccal-lingual plane) to be greater than or equal to, in degree of curvature, as clinical curvatures (mesial-distal plane) in 38% of cases. Stainless steel hand files pre-bent in one direction may not aid instrumentation of proximal curvatures and may predispose to the formation of ledges in these planes in much the same way as straight files. The files could not accurately be bent in more than one plane since proximal curvatures could not be predicted from a clinical radiograph (318). Therefore, the inability of hand and rotary instruments to contact all portions of the canal wall may leave contaminated dentin within the root canal and sustain intra- and periradicular infection.

Canal anatomy may impact the ability to disinfect a canal with hand and rotary instrumentation with or without ultrasonics. Transverse communications, or isthmuses,

between the mesiobuccal and mesiolingual canals of Type II and III mandibular molars may have a negative influence on the reduction of intracanal bacteria. These communications may occur in middle to apical portions of the root canal and may not be seen clinically even with the aid of an operating microscope. The reported occurrence of these communications in the mesial root of mandibular molars is 65% according to Kerekes and Tronstad (128), 60% according to Skidmore and Bjorndal (9) and Cambruzzi and Marshall (211), 63% according to Vertucci (11), and 33% according to Manning et al. (12). Jung et al. (325) analyzed the prevalence of isthmuses and accessory canals at the 2 mm, 3 mm, 4 mm, and 5 mm apical levels of the mesial roots of mandibular first molars. Histological sections of each mesial root were evaluated following completion of instrumentation to a size 30/.04 taper and obturation procedures. The authors found a 60% incidence of these anatomical variations at the 2 mm level, 80% incidence at the 3 mm level, 82.5% incidence at the 4 mm level, and 70% incidence at the 5 mm level. The authors concluded that instrumentation to a size 30/.04 taper does not eliminate or decrease the prevalence of isthmuses and accessory canals. Card et al. (31) detected a communication between the mesial canals in 41% of lower molars. The authors achieved negative cultures in 14 out of 15 (93%) molar canals without clinically detectable communications and in only four of seven (64%) canals with clinically detectable communications. Dalton et al. (29) and Shuping et al. (30) only instrumented and sampled the mesiobuccal canal in the mesial roots of lower molars in studies analyzing the effectiveness of nickel-titanium rotary instrumentation with saline (29) or sodium hypochlorite irrigation and placement of calcium hydroxide (30) in reducing intracanal bacteria. These authors did not account for the joining of the mesiobuccal

canal with the mesiolingual canal. This may have led to false-positive culture results in these studies due to the aforementioned problem. In our study, we did not attempt to detect communications between the mesial canals. However, no significant difference ($p = 0.853$) was found between groups with regard to the type of canal system (Table 5). Therefore, the type of canal system did not have an impact on the reduction of intracanal bacteria between Group 1 and Group 2. This may be due to the inability to retrieve bacteria from communications between the mesial canals, or to the relatively small numbers of teeth involved in the statistical analysis. Type II and III mesial roots with a communication between the mesiobuccal and mesiolingual canals may empirically impair bacterial removal, since bacteria can pass between the canals and the point of communication may be difficult to instrument. Culturing root canals with paper points also has inherent limitations, including the inability to retrieve bacteria from uninstrumented portions of the canal such as isthmuses. Even agitation of the culture medium is unlikely to retrieve these bacteria especially if the smear layer or bacterial biofilm is intact.

Although our study did not contain any detectable roots with Type IV canals, these canals may also result in decreased removal of bacteria compared to other canal types. A Type IV canal system splits into two separate canals apically, and it may be difficult to instrument both canals (2). One canal may not be instrumented if the operator is unaware of the apical canal bifurcation or is unable to gain access to the canal due to its severe divergence from the main canal. One root with a C-shaped configuration was included in Group 1 of our study. A C-shaped canal may be difficult to instrument since the ribbon-shaped canal may run the whole length of the root and exit at or near the root

apex as a single foramen or it may divide within the depth of the canal into two or more canals which exit separately (2). Furthermore, those canals usually contain many isthmuses and crevices (2). Therefore, hand and/or rotary files are unable to reach all aspects of the canal, and debris and bacteria may be left within the canal system.

Tooth type may impede bacterial removal from the root canal in an in vivo study since access to the canals may impact the ability of the provider to properly instrument the canals. First, second, and third mandibular molars may negatively influence canal cleaning. First mandibular molar mesial canals are generally curved and have more branches and ramifications from the main canals than second or third molars (2,5). Shih et al. (197) stated that irregularities in the main canal or un-treated lateral canals might contain organic material not removed by instrumentation procedures. This organic material may serve as a substrate to sustain microorganisms within infected root canals. Cavitation created through ultrasonic irrigation may serve to dislodge this material and enhance its removal. Second mandibular molar mesial canals tend to always demonstrate curvature in both buccal-lingual and mesial-distal planes (318). As previously mentioned, teeth with curvatures in both proximal and clinical planes may impede complete canal instrumentation due to preferential filing of certain canal surfaces due to limitations in flexibility with stainless steel and nickel-titanium files which can predispose the canal to ledge formation. This may further lead to inadequate irrigation of apical areas of the root canal and impair the removal of intracanal bacteria. Third mandibular molars have the most unpredictable anatomy, poor access for instrumentation, and severely curved roots (2).

Shuping et al. (30) used mandibular first and second premolars and mandibular first and second molars in a study analyzing the reduction of intracanal bacteria using rotary instrumentation and an inter-appointment dressing of calcium hydroxide. Similarly, Card et al. (31) instrumented 27 mesial roots of mandibular molars in a study analyzing the effectiveness of increased apical enlargement in reducing intracanal bacteria. McGurkin-Smith et al. (298) instrumented 19 mesial roots of mandibular molars in a study investigating the reduction of intracanal bacteria using ProFile® GT® rotary instrumentation, irrigation with 5.25% NaOCl and EDTA, and an inter-appointment dressing with calcium hydroxide. Although no ultrasonic irrigation was used in these studies, each is comparable to our study since each was an in vivo evaluation using the mesial root of mandibular molars and similar methods for obtaining the bacterial cultures were utilized. Shuping et al. (30) only instrumented and sampled the mesiobuccal root of each mandibular first and second molar. In the Card et al. (31) and McGurkin-Smith et al. (298) studies, the mesiobuccal and mesiolingual canal of each mandibular molar was instrumented, but only the mesiobuccal canal was sampled. These are important differences from our study, since the mesiobuccal and mesiolingual canal of each mandibular molar in our study was both instrumented and sampled. False-positive results may have been an issue in the Shuping et al. (30) study since only one canal was instrumented, and the results from the Card et al. (31) and McGurkin-Smith et al. (298) studies can not be extended to mesiolingual canals since only one canal was sampled. In the Card et al. (31) study, 82% of samples were bacteria-free following instrumentation and irrigation with sodium hypochlorite. In the McGurkin-Smith et al. (298) study, 53% of samples were bacteria-free following instrumentation and irrigation

with sodium hypochlorite. These values may have been lower if the mesiolingual canal had been included in the sampling procedure. Bacteria remaining in either canal may lead to treatment failure and the development of apical periodontitis. Therefore, it was our goal to recover as many remaining bacteria in either mesial canal as possible; realizing that paper point culturing techniques may not permit removal of bacteria from transverse communications between the mesial canals.

The percent of samples with negative cultures post-instrumentation in our study are fewer when compared to past studies (30,31,129,136,298) that analyzed the reduction of intracanal bacteria following hand or rotary instrumentation and irrigation with sodium hypochlorite. Shuping et al. (30) obtained three bacterial samples while instrumenting teeth to specific apical sizes using ProFile® Series 29™ nickel-titanium rotary instruments. Following initial instrumentation to an apical size of 0.216 mm for a curved molar, 0.279 mm for a straight molar and curved premolar, and 0.360 mm for a straight premolar, the mean log₁₀ CFU count was 4.03. After instrumentation to an apical size of 0.279 mm for a curved molar, 0.360 mm for a straight molar and curved premolar, and 0.465 mm for a straight premolar, the mean log₁₀ CFU count was 2.57. Following final instrumentation to an apical size of 0.360 mm for a curved molar, 0.465 mm for a straight molar and curved premolar, and 0.6 mm for a straight premolar, the mean log₁₀ CFU count was 1.27. Of the 42 teeth the authors sampled after final instrumentation, 62% were found free of bacteria. By comparison, only 35.5% of all S2 samples in our study were bacteria-free. The mean values from the Shuping et al. (30) study included results obtained from the mesiobuccal canal of lower molars and from premolars, which complicates direct comparison to our study that utilized both mesial canals of lower

molars only. The anatomy of single-rooted lower premolars may not be as complex as multi-rooted mesial canals of lower molars and this may facilitate the removal of bacteria from the root canal and therefore improve overall success. The final size of apical instrumentation in the Shuping et al. (30) study was also larger (0.360 mm for a curved molar) compared to our final apical size (0.30 mm for all molars). This too may explain the disparity between the results, since Matsumiya and Kitamura (269) showed that as canals were instrumented to larger sizes the number of intracanal bacteria decreased. An additional difference between our study and the Shuping et al. (30) study may also lie in the sampling methodology. Shuping et al. (30) only sampled the mesiobuccal canal of each molar and did not account for any remaining bacteria in the mesiolingual canal. In our study, each canal of each mesial root was sampled, which may have decreased our percentage of bacteria-free canals compared to the Shuping et al. (30) study.

McGurkin-Smith et al. (298) cultured mandibular incisors, premolars, and the mesiobuccal canal of mandibular molars following instrumentation to an apical size of 0.2 mm with ProFile® GT® rotary files and irrigation with 5.25% NaOCl and EDTA. The authors reduced the intracanal bacteria population from an initial mean of 1.01×10^7 to a post-instrumentation mean of 6.60×10^6 . Of the 31 test subjects in the McGurkin-Smith et al. (298) study, 47% cultured no bacteria following instrumentation. Our percentage of S2 samples that cultured no bacteria following instrumentation was lower than this value. The difference again could be inclusion of single-rooted teeth in the McGurkin-Smith et al. (298) study, which increased the overall results. Card et al. (31) did not report on the mean $\log_{10}$ CFU values or total CFU counts, however, the authors did report that 22 of 27 (82%) lower molar mesial canals cultured bacteria-free after

instrumentation to an apical size of 0.465 mm, and 24 of 27 (89%) lower molar mesial canals cultured bacteria-free after instrumentation to apical sizes that ranged from 0.575 mm to 0.65 mm. The difference between this study and our study is most likely due to disparities in the size of apical instrumentation. Apparently, even 6.0% NaOCl was unable to compensate for large apical sizes, since only 35.5% of canals in our study cultured bacteria-free after hand and rotary instrumentation. Similar to the Shuping et al. (30) study, Card et al. (31) and McGurkin-Smith et al. (298) also only sampled the mesiobuccal root of each molar. Our results for the percent of negative cultures may be lower due to the sampling of both the mesiobuccal and mesiolingual canals in each molar in our study. Ørstavik et al. (129) cultured teeth at each of two appointments following extensive apical reaming of the root canal to a size #45 file. The authors rendered 60% of canals bacteria-free at the end of the first appointment and 65% of canals bacteria-free at the end of the second appointment, again illustrating the value of larger apical instrumentation sizes in the reduction of intracanal bacteria. Certainly a concern with the Card et al. (31) and Ørstavik et al. (129) studies is the size of apical instrumentation; however, the safe limits of apical instrumentation have not been established. Peters et al. (136) reported a reduction in intracanal bacteria from 1.0×10^6 to 1.8×10^3 CFU/mL following instrumentation to a #35 minimum apical size and irrigation with 2% NaOCl. The authors rendered 77% of canals bacteria-free after instrumentation and irrigation. Our mean value for the number of intracanal bacteria remaining post-instrumentation and irrigation was similar to this study; however, our percentage for the number of bacteria-free canals following instrumentation and irrigation was lower, again possibly due to

differences in apical instrumentation size and the use of single-rooted teeth in the Peters et al. (136) study.

Increasing taper may improve bacterial reduction by removing greater amounts of contaminated dentin from coronal portions of the root canal. No significant differences ($p = 0.594$) were found in our study between groups with regard to instrument taper (Table 5). Therefore, this variable was not consequential in further reduction of post-instrumentation bacteria, and any differences in S2 or S3 CFU counts were not due to varying taper of the rotary instrument. Increasing instrument taper from 0.04 to 0.06 also did not improve the likelihood of obtaining a negative culture (Table 8). Our results are consistent with McGurkin-Smith et al. (298), who also demonstrated that increasing taper did not consistently render canals bacteria-free. In our study, ProFile® GT® orifice shapers in sizes #70/.12, #50/.12, and #35/.12 were used to enlarge the coronal portions of each mesial canal prior to initial bacterial sampling and toward the end of hand and rotary instrumentation. These files were used to facilitate the placement of paper points prior to initial bacterial sampling and to provide sufficient space for the ultrasonic irrigating needle to freely vibrate. The use of these larger sized files in the coronal portions of each mesial canal may have compensated for the effect of varying instrument taper. The size of apical instrumentation may also be more important than instrument taper with regard to reduction of intracanal bacteria.

Four (29-31,298) of the above studies involved the use of mesial canals of mandibular molars, and each of these was performed in vivo, as was our investigation. As stated previously, the majority of studies analyzing bacterial reduction following instrumentation procedures alone were performed in vitro on single-canal teeth.

McComb and Smith (264,265), when comparing in vitro and in vivo investigations, noted that an in vitro design allowed for a more aggressive approach that would increase debridement efficacy. Walton (215) also reported that differences in instrument manipulation would increase the effectiveness of in vitro debridement. Fairbourn et al. (267) stated that positive apical pressures present in the mouth are difficult to simulate in vitro. The authors postulated this difference in apical pressure could adversely affect canal preparation in vivo. Pineda and Kuttler (5) found mandibular molars contained a high percentage of fins and isthmuses that may complicate instrumentation procedures and the removal of bacteria from infected dental pulps. The internal anatomy of single-canal teeth may not be as complex as mandibular molars. These facts may account for the greater percent reduction in bacterial cell counts in hand and/or rotary instrumentation in vitro studies on single-canal teeth.

Determination of working length could also vary between in vivo and in vitro studies. The placement of a file 0.5 mm from the electronically determined apex in this study was based on the work of Kuttler (7) and Green (8). Peters et al. (136) also used an electronic apex locator in addition to a radiograph to confirm working length in an in vivo bacterial study. McGurkin-Smith et al. (298) established working length using the Root ZX[®] Apex Locator and radiographs. Although the Root ZX[®] Apex Locator was shown to be accurate at determining working length (280-283), it is still not as accurate as visualizing the instrument at the apex as is found with in vitro studies. In vitro working length could be accurately determined by adjusting the file length after visualizing the instrument exiting the tooth at the apical foramen, thereby increasing apical debridement efficacy as compared to in vivo length determination which cannot be

as accurate or at least results in less confidence in the accuracy of working length determination. Shuping et al. (30) and Card et al. (31) each used radiographs to determine working length during in vivo studies. However, radiographs are also not 100% accurate in determining working length (311). Inaccuracy in determining working length, either with an electronic apex locator or radiographs, may affect the number of remaining intracanal bacteria since filing short of the true apex would leave the most apical extent of the root canal contaminated. Conversely, filing beyond the true apex may result in transportation of the apical foramen and the formation of zips that may also complicate removal of bacteria from the inner portion of the apical extent of curved root canals. Shuping et al. (30), Siqueira et al. (132), and Abou-Rass and Piccinino (272) showed that NaOCl requires a certain size of canal for irrigant to reach instrumented portions of the root canal and become beneficial in bacterial reduction. Filing short, therefore, may hinder the flow of NaOCl into un-instrumented areas of the root canal, and prevent the antibacterial effects of NaOCl in the most apical extent of the root canal, which may affect the overall results. This, in turn, could lead to misinterpretation of our results where failure to eliminate bacteria is blamed on failure of the instrumentation technique instead of failure in determination of proper working length. However, every attempt was made to achieve an accurate working length in our study. Misinterpretation of working length was kept to a minimum through the combined use of the Root ZX[®] apex locator and radiographs. Therefore, inaccurate working length determination was most likely not a significant factor affecting bacterial reduction with hand and rotary instrumentation in our study.

Another factor affecting the disparity between the previously mentioned results (30,31,136) and our results may be differences in irrigating solutions. The irrigating solution used in all of these investigations (30,31,136) was sodium hypochlorite. However, Shuping et al. (30) utilized a 1.25% concentration, Card et al. (31) used a 1.0% concentration, and Peters et al. (136) used a 2.0% concentration. Our design employed the use of a 6.0% concentration. The concentration of sodium hypochlorite affects the antibacterial properties of the irrigating solution (197,198,202). Shih et al. (197) made ten-fold serial dilutions of sodium hypochlorite and found the 5.25% concentration (the maximum used) was the only effective germicide. Spångberg et al. (198) recommended the use of 0.5% sodium hypochlorite due to the cytotoxic effects of more concentrated solutions; however, the authors found the antimicrobial properties of the solution to be diminished at this diluted concentration. Harrison and Hand (202) found that dilution of 5.25% sodium hypochlorite greatly diminished its antibacterial properties. This is in contrast to Byström and Sundqvist (125), who found no difference in the antibacterial effects of 0.5% and 5.0% NaOCl during instrumentation of necrotic teeth in vivo. However, the authors only used aerobic culturing techniques and did not compare the effect of either concentration of NaOCl on facultative or obligate anaerobes. Although higher concentrations of sodium hypochlorite were shown to enhance overall antibacterial effects (197,198,202), the irrigant may not have an impact if it cannot reach contaminated areas of the root canal. Increasing the size of apical instrumentation may play a more important role than the concentration of sodium hypochlorite in decreasing the numbers of intracanal bacteria. This may further explain the disparities between our results and the results of the Shuping et al. (30) and Card et al. (31) studies where larger

apical instrumentation sizes and more dilute concentrations of sodium hypochlorite were used during preparation of the root canal.

Siqueira et al. (133) correlated increased antibacterial efficacy with high volumes of dilute irrigating solution. The authors analyzed the in vitro antibacterial efficacy of 1.0%, 2.5% and 5.25% sodium hypochlorite in extracted, single-rooted teeth inoculated with *E. faecalis*. A total of 7 mL of the selected irrigant were used for each tooth. The authors found no significant differences in the solutions tested, and suggested that regular exchange and the use of large amounts of irrigant should maintain the antibacterial effectiveness of the NaOCl solution, compensating for the effects of decreased concentration. The authors stated that frequent and copious irrigation with decreased concentrations of sodium hypochlorite may provide a chlorine reserve sufficient to significantly reduce the viable population of intracanal bacteria. However, this study was in vitro, where ecological conditions are not as diverse or complex. Our study utilized approximately 10 mL of irrigant per canal during the instrumentation phase. Shuping et al. (30), Card et al. (31), Peters et al. (136), and McGurkin-Smith et al. (298) did not report on the volume of sodium hypochlorite used during or following instrumentation. McGurkin-Smith et al. (298) only reported that each canal was irrigated for 30 minutes with 5.25% NaOCl changing to a fresh solution every 5 minutes, a technique which may not be clinically applicable. Three subjects in our study received an additional 15 mL of 6.0% NaOCl per canal following instrumentation to test the potential effect of the added volume of irrigant used during the ultrasonic irrigation procedure. None of the teeth in Group 1 that displayed bacterial growth prior to the additional irrigation were rendered bacteria-free following the added volume of irrigation. This suggests that additional

volume of irrigant may not play as prominent a factor in curved canals where preparation size could be limited. This may also be due to the inability of conventional irrigation to disperse microorganisms within the biofilm present in the root canal and leave them susceptible to attack by sodium hypochlorite. However, since we used only 6.0% NaOCl for irrigation, we cannot verify or deny the conclusions of the Siqueira et al. (133) study regarding dilute concentrations of sodium hypochlorite.

Our study showed a large, significant decrease in the population of viable bacteria in the root canal following hand and rotary instrumentation with 6.0% sodium hypochlorite irrigation. The limitations of hand and rotary instrumentation and paper point culturing techniques may not account for bacteria that were present in transverse communications between the mesiobuccal and mesiolingual canals. Hand and rotary instrumentation with 6.0% sodium hypochlorite irrigation may be shown less effective if bacteria could be retrieved from these transverse communications. Nair et al. (315) histologically demonstrated bacteria in un-instrumented areas of the root canal following instrumentation. Hand and rotary instrumentation may therefore be ineffective at removing bacteria from isthmuses, fins, and cul-de-sacs within the root canal. This may have impacted our results, since we may not have been able to retrieve bacteria from these areas of the root canal.

Dalton et al. (29) showed that hand and rotary instrumentation with sterile saline irrigation was not effective at significantly reducing the number of intracanal bacteria. Therefore, the significant reduction seen in our study between S1 and S2 was most likely due to the antibacterial properties of sodium hypochlorite combined with the physical removal of contaminated dentin using hand and rotary instrumentation. Lower irrigant

volumes may be more clinically applicable than those of our study. The optimum amount of irrigation during canal instrumentation is unknown. Harrison (274) recommended irrigation with 2 mL of solution after each instrument was used in the root canal. We modified this recommendation to irrigation after every third hand and rotary file and after the #5 Gates-Glidden bur. This was done because we felt it was more clinically applicable.

Reduction of Viable Bacteria Following Ultrasonic Irrigation (S3)

The mean CFU count in the post-ultrasonic irrigation samples (S3) for the hand file and rotary instrumentation group with ultrasonics (Group 2) was 146.7, with a range between 0 and 1.52×10^3 (Tables 3 and 4, Figures 17 and 20). Three out of 3 (100%) test teeth in Group 1 submitted for statistical analysis showed positive cultures after additional irrigation following instrumentation and only 3 out of 15 (20%) test teeth in Group 2 submitted for statistical analysis showed positive cultures after post-ultrasonic irrigation (Table 7). A trend toward significant reduction of CFU counts between groups was observed following ultrasonic irrigation, although the difference was not statistically significant ($p = 0.068$). This lack of statistical significance was due to large initial decreases in CFU counts observed between S1 and S2, which resulted in low numbers of intracanal bacteria at S2. However, a significantly ($p = 0.038$) greater percentage of canals in Group 2 cultured no bacteria at S3 than in Group 1 (Table 6). The reason for significantly greater number of canals with no bacteria in Group 2 appears to be the addition of ultrasonic irrigation after hand and rotary instrumentation. The results of our study indicated the likelihood of obtaining negative bacterial cultures is improved by using an ultrasonic irrigating needle, with a low irrigation volume, for only 1 minute per

canal. A representative photograph of an agar plate with bacterial growth obtained from post-ultrasonic irrigation specimens in our study is presented in Figure 10.

Past studies (58,138-142) have not demonstrated greater reduction in the number of intracanal bacteria or greater percent negative cultures when comparing ultrasonic irrigation to conventional means of irrigation. The lack of improved antibacterial activity in these studies may be due to a lack of sufficient ultrasonic energy to cause transient cavitation and the disruption of intracanal bacteria. The shear stress threshold value required for disruption of bacterial cell walls is 10^7 MPa (300). Ahmad et al. (58) determined that an Enac ultrasonic unit at a power setting of 3.5 generated a shear stress value of 6.4×10^6 MPa. Ahmad (140) further demonstrated that an Enac ultrasonic unit was unable to produce cavitation at a power setting of 1, 2, or 3. Ahmad et al. (45) also showed that transient cavitation did not occur with the Cavi-Endo[®] unit and Endosonic files. However, cavitation was produced when a scaler tip was inserted into the unit. This may explain the results of Martin (32), who reported elimination of greater than 90% of bacteria following short-term exposure to ultrasound with a large size probe. The 1/8-inch diameter laboratory-sized probe used by Martin (32) was capable of producing very high acoustic energy in a confined space that resulted in the elimination of a large percentage of microorganisms. However, the results of that study may not be clinically applicable, since a probe of this size could not be introduced into narrow root canals. We were able to use the MiniEndo[™] unit at a full power frequency spectrum between 25 and 32 kHz and amplitudes ranging from 5 to 250 μ m at more than 20,000 cycles per second with an ultrasonic needle that resisted breakage. Although we did not characterize the ultrasonic energy, cavitation and acoustic streaming presumably occurred since the

MiniEndo™ piezoelectric ultrasonic instrument used in our study was operated at full power (62). The only way to gauge the effect of instrument energy would be to conduct a controlled study comparing the effects of different power levels of the MiniEndo™ unit in reducing a pre-determined number of intracanal bacteria.

Contrary to Martin (32), our in vivo study utilized a 1.5-inch, 25-gauge needle that could easily be manipulated within the confines of the pulp chamber and inserted into the root canal. The ultrasonic needle was used only in the middle to coronal third of the root and did not bend around canal curves. By keeping the needle in the upper half of the canal there was less chance for dampening since the needle worked passively in the upper non-curved portion of the root canal. In our study, the ultrasonic needle was measured to determine the average needle penetration depth within the root canal. The average working length of the mesiobuccal and mesiolingual canals in Group 2 was 20.9 mm and the average needle penetration depth was 13.3 mm (Table 10). Therefore, the ultrasonic needle penetrated, on average, to 64% of working length. For comparison, the average working length of the mesiobuccal and mesiolingual canals in Group 1 was 20.8 mm. No significant difference ($p = 0.9260$) was found between Group 1 and Group 2 with regard to working length (Table 10). It was not possible to stratify the samples in Group 2 into equal groups according to needle penetration depth in order to statistically determine if penetration depth had an impact on reducing the numbers of intracanal bacteria since the needle reached similar distances in each subject who received ultrasonic irrigation and individual working lengths were similar. A further study controlling for the length of needle penetration into the root canal would be required to examine this effect in detail.

In our study, canal curvature, canal anatomy, and tooth type may have impacted ultrasonic irrigation in similar ways previously described for hand and rotary instrumentation procedures. With ultrasonic instrumentation, the ultrasonic file having to pass around the clinical and proximal curves of a canal may hinder the cavitation effect caused by an ultrasonic instrument. Contact of the instrument with the canal wall may dampen the power of the ultrasonic instrument because its motion is impeded by the curve or a tight canal as described by Ahmad et al. (47). Dampening of the ultrasonic instrument may result in insufficient energy to dislodge the microbial biofilm within infected root canals. This effect was minimized in our study since each mesial canal was sufficiently enlarged to allow free vibration of the ultrasonic irrigating needle. The ultrasonic needle was placed in each mesial canal to a point just short of binding and moved passively in an up and down motion during activation to further limit dampening of the ultrasonic instrument.

The complex root canal anatomy of mandibular molars complicates hand and rotary instrumentation as well as ultrasonic instrumentation or irrigation procedures. Transverse communications occur frequently between the mesiobuccal and mesiolingual canals of these teeth. Skidmore and Bjorndal (9) found that 60% of mandibular molar mesial canals have some form of transverse communication most commonly in the apical third of the root. Pineda and Kuttler (5) found ramifications (offshoots) of the mesial canals in 48% of first, 28% of second, and 19% of third mandibular molars. These authors also observed the majority of these ramifications were located in the apical third. Cambruzzi and Marshall (211) determined an isthmus joined the two mesial canals of mandibular molars in 60% of the cases. Vertucci (11) found transverse communications

in the mesial root of 63% of first and 31% of second mandibular molars. In approximately 75% of first and second molars this communication was found in the middle third of the root. Jung et al. (325) found a 60% incidence of isthmuses and accessory canals at the 2 mm level, 80% incidence at the 3 mm level, 82.5% incidence at the 4 mm level, and 70% incidence at the 5 mm level of the mesial roots of mandibular first molars. These isthmuses and other irregularities within the root canal system harbor tissue, bacteria, and bacterial by-products (9-11) that may lead to the persistence of endodontic disease.

These areas of the root canal were determined to be inaccessible to conventional hand and rotary instrumentation (14-18,20,22-24). Past research studying the effectiveness of ultrasound after hand and rotary instrumentation demonstrated greater canal and isthmus cleanliness values for ultrasonic instrumentation or irrigation than conventional cleaning and shaping procedures (15-17,27,62). Goodman et al. (15) compared the effect of a step-back preparation versus a step-back/three-minute ultrasound preparation using a Buffalo piezoelectric dental unit on tissue removal from the 1 mm and 3 mm levels from the mesial root canals of 60 extracted human mandibular molars. The step-back results showed 91.3% canal debridement at 1 mm and 99% at 3 mm, and 22% isthmus debridement at 1 mm and 37% at 3 mm. The ultrasound results showed 97% canal cleanliness at 1 mm and 99% at 3 mm, and 72% isthmus cleanliness at 1 mm and 92% at 3 mm. In a similar study, Lev et al. (16) compared the effect of a step-back preparation versus a step-back/one- and three-minutes ultrasound preparation using a Cavi-Endo[®] ultrasonic unit on tissue removal from the 1 mm and 3 mm levels from the mesial root canals of 59 extracted human mandibular molars. The step-back results

showed 92% canal debridement at 1 mm and 99% at 3 mm, and 39% isthmus debridement at 1 mm and 45% at 3 mm. The ultrasound results showed 97% canal cleanliness at 1 mm and 99% at 3 mm, and 82% isthmus cleanliness at 1 mm and 94% at 3 mm. The isthmus cleanliness values decreased to 44% at 1 mm and 53% at 3 mm when ultrasound was used for only 1 minute. Haidet et al. (17), in an in vivo study, compared a step-back preparation versus a step-back/ three-minute ultrasound preparation using a Cavi-Endo® ultrasonic unit on tissue removal from the 1 mm and 3 mm levels from the mesial root canals of 38 human mandibular molars. The step-back results showed 88% canal debridement at 1 mm and 99% at 3 mm, and 10% isthmus debridement at 1 mm and 69% at 3 mm. The ultrasound results showed 99% canal cleanliness at 1 mm and 3 mm levels, and 86% isthmus cleanliness at 1 mm and 94% at 3 mm. Archer et al. (27) also compared the in vivo debridement efficacy of a step-back preparation versus a step-back/three-minute ultrasound preparation on tissue removal from the 1 mm, 2 mm, and 3 mm levels from the mesial root canals of 34 human mandibular molars. Sample values at the 1 mm, 2 mm, and 3 mm levels for the step-back and step-back/ultrasonic techniques, respectively, were: canal-64% versus 92%, 81% versus 97%, and 90% versus 99%; and isthmus-2% versus 46%, 15% versus 60%, and 16% versus 83%. All of these studies showed improved canal and isthmus cleanliness values with the addition of ultrasound compared to step-back instrumentation alone.

Gutarts et al. (62) used the same ultrasonic irrigating unit as our study to histologically determine the in vivo debridement efficacy of ultrasonic irrigation following hand and rotary instrumentation in 36 vital, human mandibular molars. The mean canal cleanliness values for the hand and rotary instrumentation group were 75% at

1 mm, 97% at 2 mm, and 99% at 3 mm. The mean isthmus cleanliness values for the hand and rotary instrumentation group were 15% at 1 mm, 28% at 2 mm, and 34% at 3 mm. The mean canal cleanliness values for the hand and rotary instrumentation with ultrasonic irrigation group were 99% at 1 mm, 100% at 2 mm, and 99% at 3 mm. The mean isthmus cleanliness values for the hand and rotary instrumentation with ultrasonic irrigation group were 97% at 1 mm, 73% at 2 mm, and 94% at 3 mm. The addition of ultrasonic irrigation for only 1 minute in each mesial canal significantly increased isthmus cleanliness values at all levels tested.

Nair et al. (315) histologically illustrated the presence of sessile and planktonic bacteria in un-instrumented areas of surgical biopsies taken from the mesial roots of mandibular molars. Surgery was required in these specimens due to chronic periapical pathosis presumably arising from these remaining bacteria. In a separate study, Nair et al. (118) used light and scanning electron microscopy to examine 9 therapy-resistant asymptomatic periapical lesions removed as block biopsies during surgical treatment of the involved teeth. Microorganisms were present in the apical portion of the root canal in 6 of 9 biopsy specimens. The authors concluded the presence of microorganisms in the apical root canal may play a significant role in endodontic treatment failures. Sjögren et al. (117) showed that teeth with a negative culture at the time of obturation exhibited complete periapical healing after 5 years in 94% of the cases, whereas teeth with a positive culture showed complete healing in only 68% of cases. The persistence of bacteria in root-filled teeth therefore has a negative impact on the long-term prognosis of endodontic therapy. The use of the ultrasonic irrigating needle used in our study may help to overcome the limitations of conventional hand and rotary instrumentation

procedures in removing bacteria from isthmuses and other irregularities within the mesial roots of mandibular molars. Since Gutarts et al. (62) showed isthmus debridement values ranging from 73% to 97% in vital mandibular molars; we can postulate the ultrasonic irrigating device used in our study improved the removal of microorganisms and microbial by-products from these otherwise inaccessible areas of the root canal. Further research is required to determine the effectiveness of the ultrasonic irrigating unit used in our study in removing microorganisms and the microbial biofilm from isthmuses and other irregularities within infected root canals.

Tooth type may negatively impact ultrasonic instrumentation or irrigation of mandibular molars by limiting access to the root canals. The reader is referred to page 185 for an explanation of how first, second, and third mandibular molars may influence canal debridement. In our study, a 25-gauge, 1.5-inch needle directed at a 45° angle was used for ultrasonic irrigation. The length and position of the needle allowed for easy manipulation of the ultrasonic unit within the pulp chamber. Tooth type (first or second mandibular molar) was not a significant statistical referent in our study and did not affect the likelihood of obtaining a negative culture (Table 8). Limited access to the root canal may empirically impede bacterial removal during hand and rotary instrumentation. Ultrasonic instrumentation or irrigation procedures may serve to overcome the limitations of hand and rotary instrumentation resulting from impeded access to the root canals.

Past in vivo studies (55,138,139) evaluating the efficacy of ultrasonic instrumentation or irrigation in removing intracanal bacteria used single-rooted teeth. Sjögren and Sundqvist (55) used 31 single-rooted teeth with necrotic pulps in humans to determine the antibacterial effect of ultrasonic instrumentation. DeNunzio et al. (139)

used 44 single-rooted premolars in beagle dogs to compare ultrasonic and hand instrumentation. Barnett et al. (138) used 50 single-rooted teeth in young dogs to compare the antibacterial efficacy of sonic, ultrasonic, and hand instrumentation. Alaçam et al. (288) used single- and multi-rooted teeth in an in vivo comparison of the antimicrobial effectiveness of conventional and ultrasonic irrigation procedures. However, the study used vital teeth with a pulpal diagnosis of irreversible pulpitis. Therefore, the results of the study may be obscured, since the root canals of vital teeth with a diagnosis of irreversible pulpitis were shown by Keudell et al. (268), Shuping et al. (30), and McGurkin-Smith et al. (298) to contain low numbers of viable bacteria.

Past in vitro studies also predominantly used single-rooted teeth to evaluate the antibacterial efficacy of ultrasonic instrumentation or irrigation procedures. Weber et al. (142) evaluated the effect of passive ultrasonic activation of 2% chlorhexidine or 5.25% NaOCl irrigant on residual antimicrobial activity in 94 single-rooted extracted teeth. Huque et al. (59) evaluated ultrasonic intracanal irrigation procedures in eradicating bacteria from surface, shallow, and deep layers of root dentin using extracted human anterior teeth and mandibular premolars with a single canal. Siqueira et al. (60) analyzed the effect of irrigation with 2 mL of 4.0% NaOCl following agitation with hand files, irrigation with 2 mL of 4.0% NaOCl following ultrasonic agitation, and irrigation with NaOCl alternated with hydrogen peroxide on the disruption of *E. faecalis* in 80 extracted human canine teeth. Fegan and Steiman (141) also used extracted single-rooted human teeth with one canal to compare the antibacterial efficacy of hand and ultrasonic instrumentation to laser irradiation. Spoleti et al. (57) used human maxillary incisors, canines, and first molars to evaluate the antibacterial effect of passive ultrasonic

activation; however, the authors used only the distobuccal root of the maxillary molars. Martin (32) used 8 maxillary and 8 mandibular molars to evaluate ultrasonic disinfection of the root canal. Although maxillary and mandibular molars were used, this study was in vitro, so access to the canals was not a critical factor.

MECHANISMS OF ULTRASONIC CLEANING

The postulated reason for the increased success of Group 2 can be related to the action of the ultrasonic needle. Weller et al. (14) evaluated the use of ultrasound after hand instrumentation using a Cavitron® unit with an insert spot-welded to a 25 mm #15 stainless steel finger plugger. The authors measured the removal of radioactive gelatin from the root canals of extracted teeth and resin blocks and found that more of this medium was removed using ultrasound after hand instrumentation than when either method was used alone. This study laid the foundation for the methods which were used in the investigations of Goodman et al. (15), Lev et al. (16), Haidet et al. (17), Metzler and Montgomery (18), Archer et al. (27), Gutarts et al. (62) and the present investigation.

Ultrasonic files or needles can be utilized after hand and/or rotary instrumentation after the canal is enlarged thus enabling the energized instrument to vibrate freely. When the instrument is able to freely vibrate, the physical effects of ultrasound in a liquid medium are maximized (50). Ahmad et al. (47) believed the most important of these physical effects was acoustic streaming and not cavitation. An Enac unit was utilized in an experimental setting where cavitation would be produced. The authors previously determined the parameters required for the production of cavitation using a photometric light detection system. First, the Enac unit had to be adjusted to a power setting of at

least 3.5 (producing some cavitation). Second, and more important, the size #15 file had to vibrate at displacement amplitudes equal to at least 135 μm . Apical preparations in a molar are usually prepared to a size #30. Ahmad et al. (47) stated the size of a #40 file (0.40 mm at the tip) was required in order for the file to have enough room to move freely and for cavitation to be produced. If this statement were correct, then, in this study, cavitation may have been produced only when the energized file was moved 2-3 mm from the apical extent of the canal preparation and dampening of the file had to be at a minimum. As the energized file was passively worked in the canal during previous studies (15-17,27), it was moved 2-3 mm from the apical extent of the canal preparation. Therefore, cavitation may have been produced. Working in narrow canals, which cause dampening, the less powerful Cavi-Endo[®] unit may be as effective as the Enac unit.

Ahmad et al. (45-47) stated that acoustic streaming was produced from the sides of a file while cavitation was produced from the end point of a file. Also, acoustic streaming was produced at low and high power settings while cavitation (a more powerful force) could only be produced at high power settings in order to be effective (47). Previous studies (58,140) could not use high ultrasonic settings since this would result in file breakage. Therefore, the primary ultrasonic mechanism of bacterial reduction appeared to be acoustic streaming in these previous studies (58,140).

Our study is the first and only study, to date, to utilize an ultrasonic needle to evaluate intracanal reduction of bacteria. We believe the trend toward greater reduction of intracanal bacteria for Group 2 was due to de-aggregation of bacterial clusters within the microbial biofilm and short-term physical disruption of bacterial cell membranes as a result of cavitation. This leads to greater numbers of bacteria available for biocide attack.

Acoustic streaming, for the reasons mentioned below, may not be involved in this result even though Gutarts et al. (62) showed this phenomenon also occurred.

The needle in our study was utilized at full power (10 out of 10 setting) in the MiniEndo™ unit without breakage during the 1 minute per canal time period. No ultrasonic needles were separated in our study when the MiniEndo™ unit was used at full power. One needle was separated at the hub in the Gutarts et al. (62) study exactly at the one-minute mark when using the same ultrasonic irrigating system as our study. Archer et al. (263) showed the file holder of the Enac unit would overheat when set at a 4 of 10 power, and files would break when the unit was set at 6 of 10 power after 1.5 minutes. Therefore, there is apparently a decreased risk of breakage when using a needle versus a file for ultrasonic irrigation. The needle in our study was utilized in the middle-coronal portion of the root. Since this is a straight portion of the root and not curved, there was a decreased chance for dampening of the needle. Also, since the upper portion of the root was prepared to at least a size #70/.12 taper, the ultrasonic needle (outside diameter = 0.50 mm or #50 file) had at least 0.2 mm of space to freely vibrate. Since the needle was utilized in the upper third to half of the root and not close to the apex, the energy used to disperse the bacterial biofilm in the apical portion of the root had to come from cavitation and not acoustic streaming, since cavitation is produced from the file or needle tip (vertical plume) and acoustic streaming is produced from the file or needle sides (horizontal fashion) (45-47). Further studies are needed in order to determine if this instrument is able to produce the same type of cavitation energy at settings less than full power.

In our study, a greater percentage of canals were rendered bacteria-free following ultrasonic irrigation with 6.0% sodium hypochlorite than with conventional syringe irrigation with 6.0% sodium hypochlorite. The addition of ultrasonic energy for only 1 minute per canal was 6.98 times more likely to yield a negative culture when compared to hand and rotary instrumentation with conventional post-instrumentation irrigation. A trend toward significant reduction of CFU counts between groups was observed following ultrasonic irrigation, although the difference was not statistically significant ($p = 0.068$). This is consistent with past studies (58,138-142) that have evaluated the efficacy of ultrasonic irrigation procedures in reducing intracanal bacteria. Barnett et al. (138) evaluated the bacterial status of root canals following sonic (using the Endostar 5), ultrasonic (using the Cavi-Endo[®]) and hand instrumentation. Instrumentation with the Cavi-Endo[®] and #20 files for 3 minutes yielded no negative cultures with either 2.5% NaOCl or sterile saline as the irrigant. DeNunzio et al. (139) studied the effectiveness of hand and Endosonic instrumentation to remove a standard inoculum of bacteria from the root canal systems in the premolars of dogs. Instrumentation with the Cavi-Endo[®] and #15 to #25 Endosonic files for 1 minute each with sterile saline irrigation was not significantly greater at reducing intracanal bacteria than hand instrumentation. Fegan and Steiman (141) compared the in vitro ability of the Nd:YAG laser with saline and 5.25% NaOCl irrigation to hand and ultrasonic instrumentation using the Cavi-Endo[®] and a #15 file with saline and 5.25% NaOCl irrigation in eliminating *Bacillus stearothermophilus* from single-rooted teeth. All instrumentation times were limited to 1 minute. The authors determined that each group using sodium hypochlorite irrigation was effective in reducing the numbers of *B. stearothermophilus* from the root canal. Hand and ultrasonic

instrumentation with sterile saline irrigation did not effectively remove bacteria from the root canal. These studies (138,139,141) were not able to predictably render canals bacteria-free when utilizing ultrasonic irrigation for 1 or 3 minute time periods. The ultrasonic irrigating device used in our study consistently decreased the number of intracanal bacteria and significantly improved the likelihood of obtaining a negative culture when used for only 1 minute per canal. The lack of complete (100% success in all cases) disinfection in our study may be due to insufficient dissolution of the bacterial biofilm within the root canal. This may leave a population of bacteria not susceptible to attack by sodium hypochlorite. In addition, more time using the ultrasonic instrument may be required in each canal to completely disinfect the root canal. Further research on these topics is required.

Stumpf et al. (301) implicated cavitation as the primary mechanism responsible for the destruction of bacteria following exposure to ultrasonics. Transient cavitation is the radical oscillation and subsequent collapse of gas bubbles in the acoustic field, which results in the generation of high temperatures and free radicals. Williams (302) and Yumita et al. (303) showed that transient cavitation could damage cell walls and cell membranes through the production of high temperatures and pressures, and from the production of hydrogen and hydroxyl radicals. However, Pitt et al. (304) demonstrated this cell membrane disruption was quickly repaired; indicating that ultrasound alone was not lethal to bacteria. Qian et al. (305) also showed the effects of transient cavitation are not lethal to cells in a bacterial biofilm. Ahmad (140) proposed that cavitation had little practical importance in the disruption of root canal bacteria. Therefore, even though the ultrasonic instrument used in our study most likely produced cavitation, the effects of

transient cavitation are not the only explanation for the trend toward greater reduction of intracanal bacteria observed with ultrasonic irrigation.

The antibacterial effect of sodium hypochlorite was well documented in previous studies (197,198,200,202-206). The saponification, amino acid neutralization and chloramination reactions that occur when microorganisms and organic tissue are exposed to NaOCl are the cause for the antimicrobial and tissue dissolution properties. The antimicrobial activity is due to irreversible inactivation by hydroxyl ions and the chloramination reaction of bacterial essential enzymatic sites (196). The cell damage resulting from exposure to the generated hypochlorous acid is usually irreversible. Ultrasound may enhance the antimicrobial effects of sodium hypochlorite within the root canal by exposing bacteria to more solution. Bacteria on the surface of a biofilm within the root canal would be susceptible to the antibacterial effects of sodium hypochlorite; however, a layer of debris and dead or dying bacteria may protect the innermost cells. Joyce et al. (306) showed that low-frequency ultrasound (20 and 38 kHz) caused de-agglomeration of bacterial biofilms through the action of cavitation. This de-clumping of bacterial cells within a root canal may make individual bacteria more susceptible to attack by sodium hypochlorite. Joyce et al. (306) also demonstrated that high power ultrasound (lower frequencies) in small volumes of bacterial suspension resulted in a continuous reduction of bacterial cell numbers. The cavitation produced may also cause temporary weakening of the cell membrane making the bacteria more permeable to sodium hypochlorite (307). The de-aggregation of clusters of bacteria within the bacterial biofilm in the root canal resulting from the high power, low-frequency ultrasound, in combination with the biocidal activity of a constantly replenished supply of

sodium hypochlorite, is the most likely reason for the trend toward greater reduction of intracanal bacteria observed in our study. The ultrasonically energized needle may also increase the temperature of the sodium hypochlorite irrigating solution. Cunningham and Joseph (201) determined the time required to eliminate bacterial growth was much less at body temperature (37°C) than at room temperature (32°C). Therefore, heated sodium hypochlorite may also explain the trend toward greater reduction of intracanal bacteria observed in our study.

From our viewpoint, it was highly unlikely that additional irrigation with 6.0% NaOCl increased the probability of obtaining a negative culture in our study. Clinically, intracanal microorganisms exist as a complex milieu known as a biofilm. A biofilm was defined by Wilson (314) as an aggregation of bacteria associated with a surface, embedded in an extra-cellular matrix of polysaccharide, which differ greatly in phenotype when compared to their planktonic (free-floating) counterparts and are less susceptible to antimicrobial killing. Bacterial biofilms are highly organized structures consisting of mushroom-shaped clumps of bacteria bound together by carbohydrate moieties and perforated by water channels that deliver nutrients and remove wastes (322). Exopolysaccharide production by biofilm bacteria consists of variable saccharide lectins and enhances resistance to both environmental stress and antimicrobial agents. The mesh size and chemical characteristics of the microbial exopolysaccharides limit penetration and reactivity of antimicrobial agents within the biofilm (321). Dense living biofilms and associated polymers present a molecular radius-dependent barrier to diffusion of antimicrobial agents (322). Spratt et al. (323) demonstrated this concept using a laboratory model of single-species biofilms. Large numbers of viable bacteria remained

following a fifteen-minute contact time with sodium hypochlorite or povidone-iodine. Nair et al. (315) determined that bacteria in instrumented and un-instrumented areas of the root canal existed as matrix-embedded multi-leveled structures attached to the dentinal pulpal walls and also as planktonic cells, aggregates, or co-aggregates of the species suspended in the fluid phase of the necrotic, infected root canal. This matrix-embedded collection of multi-species organisms exists in microecosystems that were immobilized on dentinal surfaces (316) as a biofilm, and were resistant to host defenses and chemotherapeutic agents (315).

Sessile biofilm communities can give rise to non-sessile planktonic individuals that can multiply and disperse and cause acute clinical symptoms (324). Numerous acute and chronic human infections involve species-specific biofilms. For example, dental caries results from acidogenic Gram-positive cocci biofilm formation, periodontitis results from Gram-negative anaerobic oral bacterial biofilms, skin suture tract infections are caused by biofilms of *S. epidermidis* and *S. aureus*, and native valve endocarditis results from viridans group streptococcal biofilms (324). The nature and composition of biofilm formation in endodontic disease has not been fully elucidated. Noiri et al. (327) examined the morphological characteristics of bacterial biofilms from 6 teeth and 5 extruded gutta-percha points associated with refractory apical periodontitis.

Extraradicular biofilms were found in 9 out of 11 specimens. The gutta-percha surface was covered with a glycocalyx-like structure and filamentous or spirochete-shaped bacterial cells were aggregated in most parts of the extraradicular area. Most periapical root surfaces were covered by a biofilm consisting of long rods and filamentous shaped bacteria. Further research is required to reveal the most common bacterial isolates and

polysaccharide matrix components of root canal biofilms. Nevertheless, it is highly unlikely syringe irrigation with 6.0% sodium hypochlorite would remove the bacterial biofilm in complex root canal systems such as the mesial roots of mandibular molars. Failure to dislodge the microbial biofilm within infected teeth may decrease the success rate of endodontic therapy, since remaining sessile or planktonic bacteria may repopulate the root canal and result in acute clinical symptoms or chronic periapical pathosis. Mechanical dislocation of these largely sessile bacteria and subsequent exposure to antimicrobial solutions are required to further reduce the intracanal bacterial load. Three subjects in our study were subjected to additional irrigation without ultrasound to test this theory. No canals with growth following the initial irrigation procedure were rendered bacteria-free following the additional irrigation procedure (Table 11) suggesting that additional irrigation without ultrasound could not dislocate the bacterial biofilm.

The optimum volume of irrigation for use in ultrasonic instrumentation following hand instrumentation is unknown. The volume of sodium hypochlorite appeared not to have an impact on the reduction of intracanal bacteria in our study, since none of the teeth in Group 1 that displayed bacterial growth prior to additional irrigation were rendered bacteria-free following the added volume of irrigation. Therefore, the data at S2 for Group 1 was used to compare the data at S3 for Group 2. This finding is similar to Windley et al. (320) who showed that irrigation with 10 mL of 1.25% NaOCl without instrumentation was unable to consistently provide a bacteria-free environment in immature dog teeth with open apices. Following irrigation, the authors recovered bacteria from 27 of 30 (90%) samples.

The use of a traditional high volume continuous ultrasonic irrigation system with solution delivery and replenishment at the canal orifice presents difficulties when used clinically. Previous studies (14-18,33-44,46,50,51,244,251,294) using continuous irrigation in conjunction with ultrasonic instrumentation reported problems in the in vivo setting because the irrigating solution was applied using a reservoir in the ultrasonic unit (14,17,33-44,46,50,51,244,251,294) or an intravenous bag (15,16,18). If an intravenous bag is used, a special hookup is needed to suspend the bag and a constant flow rate cannot be assured. Three studies (15,16,18) were performed in vitro where the irrigating solution was easily evacuated. Haidet et al. (262) reported that it was necessary to reinforce the rubber dam with Cavit[®] to prevent leakage. Even with this precaution, irrigating solution leaked into the patient's mouth in two cases. When used in small volumes with delivery directly into the canal, as was used in our study, solution evacuation was easy to control. Our system could be even easier to control by a single operator, with no assistant, if a mechanical pump was used to express the irrigant instead of the operator expressing it by hand. Haidet et al. (262) noted the reservoir within the Cavi-Endo[®] unit required replenishing between instrumentation of the mesial canals. This step could cause an awkward interruption during a clinical procedure. The use of an IV bag to dispense the irrigant poses another problem. The rate of irrigation could be hard to precisely control since only gravity and a flow-control clamp would control irrigation rate. In our study, the operator controlled flow-rate, and replacing the irrigant filled syringes proved to be quick and efficient due to the Luer-Lok connectors. In addition, our results were obtained by utilizing ultrasonic irrigation for only 1 minute per

canal unlike previous studies (15-17,27) that required 3 minutes of ultrasonic irrigation per canal to improve debridement results.

Clinically, the addition of ultrasonic instrumentation may reduce the impact of operator skill following hand instrumentation. Although no studies have compared the skill of the operator in reducing intracanal bacteria, Goodman et al. (15) found significant differences in canal cleanliness after hand instrumentation when two different operators performed the procedure. This finding supported the conclusion of Littman (308), which stated the operator had more influence on canal cleanliness than the technique. However, when examining the step-back and ultrasound cleanliness values in the Goodman et al. (15) study, no difference was found between the two operators. Therefore, the use of ultrasound after hand instrumentation was shown to reduce the effect of intra-operator differences. A single operator was used in our study; therefore, we cannot conclude if the addition of ultrasound will level the differences between operators. However, we postulate the ultrasonic device used in our study should help render a greater percentage of canals bacteria-free regardless of operator.

Throughout this discussion and this thesis, the term “bacteria-free” was used loosely. Culturing of infected root canals using a paper point technique has certain limitations that may not reflect the true microbial status of the root canal. Paper point sampling can only recover bacteria from the instrumented portions of the root canal. Nair et al. (118,315,317) on several occasions showed using histological sections of biopsy material that microorganisms may be in locations that are inaccessible to sampling. Certain bacterial species, especially *E. faecalis*, may penetrate dentinal tubules to distances approaching 250 μm (119-121). Ultrasonic energy may not be sufficient to

dislodge these bacteria from unique ecological niches such as dentinal tubules. Inter-appointment dressings with calcium hydroxide or irrigation with alternative medicaments such as MTAD™ (170) may be required to completely disinfect root canals prior to obturation. Regardless, the ultrasonic device used in our study did consistently reduce the numbers of bacteria from those obtained with hand and rotary instrumentation with conventional irrigation. The number of remaining bacteria necessary to cause persistent infection and failure of endodontic treatment is unknown. Culture reversals are known to occur (312,313); therefore, even small numbers of bacteria left may re-populate the root canal and result in treatment failure. Culturing the root canals 2-4 weeks after collection of S3 would better show if the canals in our study were truly bacteria-free. The theory of entombing remaining bacteria within obturation material also lacks scientific support. The 4 to 6 year success rate of teeth with pre-operative periapical radiolucencies is 81%, compared to 94% success on teeth without pre-operative periapical radiolucencies (326). This suggests the presence of bacteria within the root canal negatively impacts the success rate of endodontic therapy on necrotic teeth. Reducing viable bacterial counts as much as possible should, empirically, increase the success rate of endodontic therapy. Further research in this area is required.

ELIMINATION OF *E. FAECALIS* WITH AND WITHOUT THE USE OF ULTRASOUND

Previous studies (102,103,143,160-163) have associated *E. faecalis* with failure of endodontic therapy. Therefore, in our study we compared the ability of hand and rotary instrumentation with and without ultrasonic irrigation to eliminate viable intracanal *E.*

faecalis using 16S rDNA polymerase chain reaction. Only agar plates demonstrating viable bacterial colonies prior to instrumentation and after hand and rotary instrumentation for teeth in Group 1 or after hand and rotary instrumentation and hand and rotary plus ultrasonic irrigation for teeth in Group 2 were chosen for PCR analysis. Using PCR, *E. faecalis* was detected in 3 out of 4 (75%) initial (S1) samples in Group 1 and 4 out of 5 initial (S1) samples in Group 2 (Table 9). The occurrence of *E. faecalis* in past studies (155-158,187,189) ranged from 0 to 30% in infected, necrotic teeth with primary apical periodontitis. The occurrence of *E. faecalis* is more prevalent in cases of failed root canal therapy. Past studies (102,103,160) indicated the presence of *E. faecalis* in up to 75% of cultures from teeth with refractory apical periodontitis. De Sousa et al. (155) using a paper point sampling technique and culture methods for identification failed to isolate any species of *Enterococcus* from the root canals of 30 necrotic teeth with associated periapical abscesses. Engström (156) used differential culture methods to isolate enterococci from 12% of culture-positive teeth at the onset of treatment of necrotic root canals. Siqueira et al. (157) using checkerboard DNA-DNA hybridization isolated *E. faecalis* from 7.5% of the root canals from 53 single-rooted necrotic teeth prior to treatment. Mejare (158) used microbial culture methods to demonstrate the presence of enterococci in 30% of 612 root canals with a diagnosis of primary apical periodontitis. Rôças et al. (187) detected *E. faecalis* in 18% of clinical specimens from teeth with primary apical periodontitis using PCR. Baumgartner et al. (189) isolated *E. faecalis* from 16% of specimens obtained from Portland, OR and in 17% of samples from Rio de Janeiro, Brazil using PCR. Our values for the presence of *E. faecalis* prior to instrumentation of 75% for teeth in Group 1 and 80% for teeth in Group 2 is higher than

these studies, most likely due to the low numbers of teeth in the analysis. This number may be reduced if PCR could have been performed on more clinical samples. We did not use PCR if no growth was present post-instrumentation; therefore, colonies of *E. faecalis* may or may not have been present on initial samples where no growth was observed following hand and rotary instrumentation, leaving our value of 75% slightly skewed.

In our study, hand and rotary instrumentation with intermittent 6.0% sodium hypochlorite irrigation eliminated colonies of *E. faecalis* in 1 out of 4 (25%) post-instrumentation (S2) samples from subjects in Group 1 and 4 out of 5 (80%) post-instrumentation (S2) samples in Group 2 (Table 9). Between Group 1 and Group 2, hand and rotary instrumentation with intermittent 6.0% sodium hypochlorite irrigation eliminated *E. faecalis* in 5 out of 9 (56%) samples. Hand and rotary instrumentation followed by ultrasonic irrigation with 6.0% sodium hypochlorite eliminated colonies of *E. faecalis* in 1 out of 2 (50%) samples (Table 9). These values may be reduced if we had included samples with no growth post-instrumentation as containing no *E. faecalis*. However, since we did not use PCR on initial samples with no growth post-instrumentation, we could not conclude that hand and rotary instrumentation with or without ultrasonic irrigation was capable of removing *E. faecalis* from the root canal. Representative photographs of agarose gels used for detection of *E. faecalis* are found in Figures 11-15.

Past studies (167,170) have indicated the relative refractoriness of *E. faecalis* to conventional instrumentation and irrigation procedures. Peciuliene et al. (167) isolated *E. faecalis* from 6 of 10 symptom-free and root-filled teeth following chemomechanical preparation of the root canal. Shabahang and Torabinejad (170) recovered *E. faecalis*

from 6 of 15 extracted single-canal teeth following cleaning and shaping and a five-minute irrigation with 1.3% sodium hypochlorite, and from 7 of 15 teeth following cleaning and shaping and a five-minute irrigation with 5.25% sodium hypochlorite. Our study confirms these studies and further demonstrates the inability of hand and rotary instrumentation with NaOCl irrigation to effectively eliminate *E. faecalis* from the root canal. This inability may be due to lack of contact between the 6.0% sodium hypochlorite used in our study and the *E. faecalis* cell, since *E. faecalis* was shown to penetrate dentinal tubules up to 250 μm (119-121). Evans et al. (168) showed that even dilute concentrations of sodium hypochlorite are rapidly lethal to cells of *E. faecalis* in vitro; therefore our 6.0% NaOCl solution should have eliminated *E. faecalis* if the solution came into contact with the bacterial cell. The ultrasonic irrigation device used in our study may be more effective against *E. faecalis* if it were used for longer periods of time, with heated sodium hypochlorite, or with alternative irrigants such as chlorhexidine. Further research is required to determine the effects of each of these variables.

Calcium hydroxide preparations are also reportedly ineffective at removing *E. faecalis* from infected root canals (161,162,165,166); possibly due to a functioning proton pump responsible for pumping cations into the cell to lower the internal pH (168). Byström et al. (126) and Evans et al. (168) showed that *E. faecalis* does not survive at a pH of 11.5 or greater, yet it can survive at a pH below 11.5. At a pH of 11.1, Evans et al. (168) showed that calcium hydroxide decreased counts of *E. faecalis* from 2.3×10^9 to 4.8×10^{-1} after 15 minutes and to 4.1×10^{-1} after 30 minutes. With calcium hydroxide at a pH of 11.5, the number of cells was significantly further reduced to 2.6×10^{-4} after 15 minutes and 1.3×10^{-6} after 30 minutes (168). Shabahang and Torabinejad (170) studied

the effect of a mixture of a tetracycline isomer, citric acid, and the detergent Tween 80 (BioPure™ MTAD™) on *E. faecalis*-infected root canals of 85 extracted single-rooted human anterior teeth and showed this mixture to be effective in eliminating *E. faecalis* in 15 out of 15 (100%) teeth. Although some studies show an enhanced ability to reduce viable cultures of *E. faecalis* using new irrigants such as MTAD™ (170) or by combining calcium hydroxide with other medicaments or vehicles for delivery (164-166), these systems require extended treatment times and have not been tested in vivo, where conditions are ecologically diverse and clinically more challenging. Furthermore, Portenier et al. (299) showed that *E. faecalis* resistance to various irrigants was dependent on the physiologic state of the bacteria, with cells in starvation phase more resistant and able to resist the effects of medicaments for 10 minutes. This further complicates the removal of *E. faecalis* from the root canal, since the physiologic state of bacteria is not known during instrumentation.

It is important to note that we did not investigate the ability of hand and rotary instrumentation with intermittent 6.0% sodium hypochlorite irrigation or with ultrasonic irrigation to reduce intracanal bacteria in cases of failed endodontic treatment. Our study was limited to mandibular molars with a diagnosis of primary apical periodontitis, where populations of *E. faecalis* are not as abundant as in cases of failed treatment with secondary apical periodontitis. It is difficult to conjecture the effectiveness of our instrumentation or ultrasonic irrigation regimen in cases of secondary apical periodontitis; however, based on previous studies (102,126), we can hypothesize that instrumentation with NaOCl without the use of inter-appointment dressings such as calcium hydroxide would be relatively ineffective in removing intracanal populations of

E. faecalis. Further studies using the ultrasonic irrigation system are required to determine the effectiveness of ultrasonic irrigation in removing *E. faecalis* from teeth with primary and secondary apical periodontitis.

CHAPTER 6

SUMMARY AND CONCLUSIONS

This in vivo study compared the antibacterial efficacy of a hand and rotary instrumentation technique and conventional irrigation with 6.0% sodium hypochlorite to a hand and rotary instrumentation technique followed by a one-minute irrigation with an ultrasonic irrigating needle in necrotic, infected mesial root canals of human mandibular molars. A MiniEndo™ dental unit operated at the maximum power level was used as the ultrasonic source.

Experimental teeth were randomly divided between two groups. Group 1 consisted of 17 mesial roots initially instrumented by hand with K-files followed by ProFile® GT® nickel-titanium rotary files using a crown-down technique. Group 2 consisted of 16 mesial roots instrumented in the same manner, followed by 1 minute of ultrasonic irrigation per canal. Both groups were irrigated intermittently with 6.0% sodium hypochlorite during the hand and rotary phase of instrumentation. Group 1 and Group 2 received 15 mL of sodium hypochlorite irrigation using a conventional syringe following completion of hand and rotary instrumentation. Group 2 also received an additional 15 mL per canal of constant irrigation using 6.0% sodium hypochlorite through the ultrasonic needle during the ultrasonic phase of cleaning. Three teeth in

Group 1 received an extra 15 mL of sodium hypochlorite per canal to control for the volume of irrigation.

Bacteria were sampled from both mesial canals for each tooth prior to instrumentation, following hand and rotary instrumentation, and following the additional irrigation procedure for the control teeth in Group 1 and ultrasonic irrigation for teeth in Group 2. Samples were taken using a paper point transfer protocol following dispersal of the microorganisms in reduced transport fluid within the root canal. Each sample was transported in reduced transport fluid to a microbiology laboratory where 10-, 100-, and 1000-fold serial dilutions were completed and 0.25 mL aliquots of each dilution were plated on reduced blood agar. Colony-forming units were counted following a seven-day anaerobic incubation period. The presence of *E. faecalis* was also determined using PCR on selected teeth demonstrating initial growth and growth following hand and rotary instrumentation or ultrasonic irrigation.

Mean initial $\log_{10}$ CFU counts were 5.3 ± 1.1 for Group 1 and 5.5 ± 0.8 for Group 2. Mean post-instrumentation and syringe irrigation $\log_{10}$ CFU counts were 1.6 ± 1.7 for Group 1 and 2.3 ± 1.8 for Group 2. Statistical analysis using a dependent t-test indicated that mean $\log_{10}$ CFU counts were significantly ($p < 0.0001$) reduced from initial $\log_{10}$ CFU counts following hand and rotary instrumentation and conventional irrigation with 6.0% sodium hypochlorite. Also, there were consistently low standard deviations relative to the mean in post-instrumentation samples. The mean post-ultrasonic irrigation CFU count for Group 2 was 146.7. A trend toward significant reduction of CFU counts was observed between groups following the addition of ultrasonic irrigation, although the results were not statistically different ($p = 0.068$). The lack of significance was due to a

large initial decrease in the mean CFU count following hand and rotary instrumentation with syringe irrigation. However, a significantly ($p = 0.038$) higher percentage of canals cultured no bacteria following ultrasonic irrigation (80%) than following hand and rotary instrumentation with conventional irrigation alone (35.5%). Logistic regression analysis indicated that ultrasonic irrigation was 6.98 times more likely to yield a negative culture than hand and rotary instrumentation with conventional irrigation. No canals in Group 1 that exhibited growth following the initial instrumentation and irrigation procedure were rendered bacteria-free following the additional irrigation procedure, indicating the addition of ultrasound and not additional irrigation was responsible for further elimination of intracanal bacteria. Statistical analysis was not performed between Group 1 and Group 2 in the removal of *E. faecalis* from the root canal since the numbers of teeth in each group were too small to form statistical conclusions.

A consistent decrease in CFU counts was observed following the addition of ultrasonic irrigation, and a higher percentage of canals cultured bacteria-free following ultrasonic irrigation than with hand and rotary instrumentation with conventional irrigation. Ultrasonic irrigation with 6.0% sodium hypochlorite was 6.98 times more likely to yield a negative culture than hand and rotary instrumentation without ultrasound. Achieving negative cultures following treatment of necrotic, infected mandibular molars may improve the long-term endodontic success of these teeth, and the ultrasonic irrigation system used in our study may assist in reaching this goal. The use of the ultrasonic device may eliminate the need for multi-appointment endodontic therapy, making the device practical and time efficient. However, inter-appointment dressings

with calcium hydroxide or alternative irrigants may still be required to predictably eliminate as many intracanal bacteria as possible.

APPENDIX A

TABLES

	GROUP 1	GROUP 2
Subjects	17	16
Curvature *		
Moderate (0-25°)	9/17 (53%)	9/16 (56%)
Severe (>25°)	8/17 (47%)	7/16 (44%)
Canal Type		
I	0/17 (0%)	1/16 (6%)
II	8/17 (47%)	8/16 (50%)
III	8/17 (47%)	7/16 (44%)
IV	0/17 (0%)	0/16 (0%)
C-shaped	1/17 (6%)	0/16 (0%)
Tooth Type		
First Molars	11/17 (65%)	12/16 (75%)
Second Molars	6/17 (35%)	4/16 (25%)
Third Molars	0/17 (0%)	0/16 (0%)
Size of Radiolucency		
0-16 mm ²	7/17 (41%)	5/16 (31%)
>16 mm ²	10/17 (59%)	11/16 (69%)

(*) Values for curvature, canal type, tooth type, and size of radiolucency represent percentages of all subjects in individual groups.

Table 1. Curvature, canal type, tooth type, and size of radiolucency for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

Variable	N*	Mean	SD	Median	Q-range	Min.	Max.	P†
Patient Age (years)								
Group 1	16	34.8	13.4	32.0	19.5	20	64	0.2679
Group 2	15	40.3	14.8	42.0	22.0	19	66	
Lesion Area (mm²)								
Group 1	16	33.6	32.6	24.5	35.5	6	132	0.5266
Group 2	15	36.1	25.2	28.0	38.0	5	90	
Curvature (degrees)								
Group 1	16	20.4	14.1	17.5	19.0	3	56	0.4057
Group 2	15	23.1	13.7	22.0	18.0	7	61	
Working Length (mm)								
Group 1	16	20.8	1.8	21.0	2.0	18	24	0.9260
Group 2	15	20.9	2.7	20.0	3.0	17	23	

(*) One subject from each group was eliminated from the statistical analysis due to outlying canal type.

(†) Exact Mann-Whitney-Wilcoxon Test.

Table 2. Between group differences for age, lesion area, canal curvature, working length, and associated p-values for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

Variable	N*	Mean	SD	Median	Q-range	Min.	Max.	P†
S1								
Group 1	16	1098527.5	1801937.1	186000	1107600	240	5440000	0.3845
Group 2	15	1074826.7	1238074.8	604000	2128000	28400	4080000	
S2								
Group 1	16	12171.3	44881.4	50.0	140.0	0	180000	0.0933
Group 2	15	10660.0	24296.9	360.0	4000.0	0	88000	
S3								
Group 1†	16	12171.3	44881.4	50.0	140.0	0	180000	0.0679
Group 2	15	146.7	410.0	0.0	0.0	0	1520	

(*) One subject from each group was eliminated from the statistical analysis due to outlying canal type.

(†) Exact Mann-Whitney-Wilcoxon Test.

(‡) The same values for S2 and S3 in Group 1 were used for statistical analysis since additional irrigation did not increase antibacterial efficacy.

Table 3. Between group differences for initial CFU count (S1), post-instrumentation CFU count (S2), post simple/ultrasonic CFU count (S3), and associated p-values for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

Variable	N	Mean	SD	Min.	Max.	P*
Group 1						
S1	17	5.3	1.1	2.4	6.7	<0.0001
S2	17	1.6	1.7	0.0	5.3	
Group 2						
S1	16	5.5	0.8	4.5	6.6	<0.0001
S2	16	2.3	1.8	0.0	4.9	

(*) Dependent T-test.

Table 4. Intra-group differences for log₁₀ initial CFU count (S1) versus log₁₀ post-hand and rotary instrumentation CFU count (S2) and associated p-values.

Variable	N		Percent [*]		P [†]
	<u>Group 1</u>	<u>Group 2</u>	<u>Group 1</u>	<u>Group 2</u>	
Gender					
Male	9	11	29.0	35.5	0.3205
Female	7	4	22.6	12.9	
Tooth Type					
First Molar	11	12	35.5	38.7	0.4744
Second Molar	5	3	16.1	9.7	
Canal Type					
II	8	8	25.8	25.8	0.8528
III	8	7	25.8	22.6	
Final Taper					
0.04	9	7	29.0	22.6	0.5936
0.06	7	8	22.6	25.8	

(*) Percent of 31 subjects used for statistical analysis.

(†) Chi-Square Test.

Table 5. Between group differences for gender, tooth type, canal type, final taper, and associated p-values for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

Variable	N		Percent [*]		P [†]
	<u>Group 1</u>	<u>Group 2</u>	<u>Group 1</u>	<u>Group 2</u>	
Bacteria Present (S2)					
Yes	9	11	29.0	35.5	0.3205
No	7	4	22.6	12.9	
Bacteria Present (S3)					
Yes	9	3	29.0	9.7	0.0384
No	7	12	22.6	38.7	

(*) Percent of 31 subjects used for statistical analysis.

(†) Chi-Square Test.

Table 6. Between group differences for positive cultures at S2 and S3 for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

	GROUP 1	GROUP 2	TOTAL
N	16	15	31
S1 Positive Cultures*	16 (100%)	15 (100%)	31 (100%)
S2 Positive Cultures	9 (56%)	11 (73%)	20 (65%)
S3 Positive Cultures	3/3 (100%) [†]	3 (20%)	6 (32%)

(*) Percent of 16 subjects in Group 1 and 15 subjects in Group 2 used for statistical analysis.

(†) Data from subjects in Group 1 who received additional irrigation following hand and rotary instrumentation.

Table 7. Number of teeth showing positive S1, S2, and S3 cultures for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

Parameter	Referent	SE	Adj. OR	Lower_{.95}	Upper_{.95}
Taper	0.06 vs. 0.04	0.647	2.018	0.576	7.544
Tooth Type	Second vs. First Molar	0.724	0.338	0.073	1.313
Canal Type	III vs. II	0.629	1.456	0.419	5.090
Method of Instrumentation	No ultrasound vs. ultrasound	0.633	6.979	2.148	26.385

Table 8. Logistic regression indicating the likelihood of bacterial presence at S3 associated with instrument taper, tooth type, canal type, and method of instrumentation with standard error (SE), adjusted odds ratios, and 95% confidence intervals.

	GROUP 1	GROUP 2
S1 Positive <i>E. faecalis</i>	3/4 (75%)	4/5 (80%)
S2 Positive <i>E. faecalis</i>	1/4 (25%)	4/5 (80%)
S3 Positive <i>E. faecalis</i>	-	1/2 (50%)

Table 9. Number of teeth showing positive S1, S2, and S3 *E. faecalis* cultures for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

	GROUP 1	GROUP 2	P*
Working Length (mm)	20.8	20.9	0.9260
Needle Penetration (mm)	-	13.3 (63.6%)	-

(*) Exact Mann-Whitney-Wilcoxon Test

Table 10. Average working lengths and associated p-values for Group 1 and Group 2 and average ultrasonic needle penetration depth for Group 2.

	GROUP 1
N	3
S1	284000
S2	3565
S3	720
Number of Samples with Growth Post-Instrumentation	3
Number of Samples with Growth Post-Additional Irrigation	3

Table 11. Initial, post-instrumentation, post-additional irrigation, number of samples with growth post-instrumentation, and number of samples with growth post-additional irrigation for samples in Group 1.

APPENDIX B

FIGURES

Figure 1. Perspective view of ultrasonic device.

Figure 2. Perspective view of ultrasonic device during ultrasonic irrigation within a root canal.

Figure 3. Schematic representation of the prokaryotic ribosomal operon and the locations and orientations of the primers used and the resulting DNA fragment. The variable spacer region is located between the 16S and 23S genes. Species-specific ENFE forward primers and universal L189 reverse primers were used to generate rDNA spacer region fragments specific for *E. faecalis* using PCR. The binding position of each primer is indicated below the primer name in parentheses. The expected PCR amplicon size was approximately 718 bp.

Figure 4. Representative blood agar plate from subject 578483 (S1, 1/1000 dilution) used for quantification of colony-forming units. The arrows indicate representative colonies used for quantification of colony forming units.

Figure 5. Representative blood agar plate from subject 550273 (S1, 1/1000 dilution) used for quantification of colony-forming units. The arrows indicate representative colonies used for quantification of colony forming units.

Figure 6. Representative blood agar plate from subject 453062 (S1, 1/1000 dilution) used for quantification of colony-forming units. The arrows indicate representative colonies used for quantification of colony forming units.

Figure 7. Representative blood agar plate from subject 967021 (S1, 1/100 dilution) used for quantification of colony-forming units. The arrows indicate representative colonies used for quantification of colony forming units.

Figure 8. Representative blood agar plate from subject 453062 (S2, undiluted) used for quantification of colony-forming units. The arrows indicate representative colonies used for quantification of colony forming units.

Figure 9. Representative blood agar plate from subject 444244 (S2, 1/10 dilution) used for quantification of colony-forming units. The arrows indicate representative colonies used for quantification of colony forming units.

Figure 10. Representative blood agar plate from subject 967021 (S3, 1/10 dilution) used for quantification of colony-forming units. The arrows indicate representative colonies used for quantification of colony forming units.

Figure 11. rDNA amplified directly from blood agar plates. Markers are *Eco*R1 and *Hind*III digestion products of bacteriophage lambda DNA. Lane 2 represents DNA amplified from *E. faecalis* strain ATCC 29212. Lanes 3-14 on the top half of the gel represent DNA amplified from an S1 agar plate of subject 404311, with clear bands at 718 bp demonstrating the presence of *E. faecalis*. Lane 3 on the bottom half of the gel represents DNA amplified from an S2 agar plate of subject 404311, with a clear band at 718 bp demonstrating the presence of *E. faecalis*.

Figure 12. rDNA amplified directly from blood agar plates. Markers are *Eco*R1 and *Hind*III digestion products of bacteriophage lambda DNA. Lane 2 represents DNA amplified from *E. faecalis* strain ATCC 29212. Lanes 3-11 on the top half of the gel represent DNA amplified from an S1 agar plate of subject 499158, with clear bands at 718 bp demonstrating the presence of *E. faecalis*. Lanes 2 and 3 on the bottom half of the gel represent DNA amplified from an S2 agar plate of subject 499158, with clear bands at 718 bp demonstrating the presence of *E. faecalis*.

Figure 13: rDNA amplified directly from blood agar plates. Markers are *Eco*R1 and *Hind*III digestion products of bacteriophage lambda DNA. Lane 2 represents DNA amplified from *E. faecalis* strain ATCC 29212. Lanes 3-14 on the top half of the gel represent DNA amplified from an S1 agar plate of subject 444244, with no detectable *E. faecalis*. Lanes 2-6 on the bottom half of the gel represent DNA amplified from an S2 agar plate of subject 444244, with no detectable *E. faecalis*.

Figure 14. rDNA amplified directly from blood agar plates. Markers are *Eco*R1 and *Hind*III digestion products of bacteriophage lambda DNA. Lane 2 represents DNA amplified from *E. faecalis* strain ATCC 29212. Lanes 3-14 on the top half of the gel and lanes 2-4 on the bottom half of the gel represent DNA amplified from an S1 agar plate of subject 550273, with clear bands at 718 bp demonstrating the presence of *E. faecalis*.

Figure 15. rDNA amplified directly from blood agar plates. Markers are *Eco*R1 and *Hind*III digestion products of bacteriophage lambda DNA. Lane 2 represents DNA amplified from *E. faecalis* strain ATCC 29212. Lanes 3-14 on the top half of the gel and lanes 2-4 on the bottom half of the gel represent DNA amplified from an S2 agar plate of subject 550273, with clear bands at 718 bp demonstrating the presence of *E. faecalis*.

Figure 16. Graph displaying S1, S2, and S3 CFU counts by observation for Group 1 (hand and rotary instrumentation without ultrasound).

Figure 17. Graph displaying S1, S2, and S3 CFU counts by observation for Group 2 (hand and rotary instrumentation with ultrasound).

Figure 18. Graph displaying S1 CFU counts by observation for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

Figure 19. Graph displaying S2 CFU counts by observation for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

Figure 20. Graph displaying S3 CFU counts by observation for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

Figure 21. Number of samples with positive cultures at S1, S2, and S3 for Group 1 (hand and rotary instrumentation without ultrasound) and Group 2 (hand and rotary instrumentation with ultrasound).

APPENDIX C
PATIENT CONSENT FORM

THE OHIO STATE UNIVERSITY

Protocol No. 2004H0116

CONSENT TO INVESTIGATIONAL TREATMENT OR PROCEDURE

I, _____, hereby authorize or direct Dr. John Nusstein and associates or assistants of his choosing to perform the following treatment or procedure (describe in general terms),

I have been asked to participate in this research study because I decided to have a root canal. A total of 80 people will be asked to participate in this study.

upon: _____
(myself or name of subject)

The experimental (research) portion of the treatment or procedure is:

To test two different methods in cleaning molar root canals, and determine which method eliminates more bacteria.

Two of the root canals in my tooth will be cleaned with small files, or with a combination of small files and an ultrasonic dental unit. I will then have my root canals sampled for bacteria (germs). Bacterial sampling is usually not performed during a root canal. A small amount of fluid used to transport bacteria will be placed in my tooth and the fluid will be agitated with small files to disperse the bacteria within the fluid. Paper cones will then be used to absorb the fluid and collect the bacteria in my tooth.

My tooth will be cleaned using a standard treatment with small files, or with an experimental treatment using small files and an ultrasonic dental unit. Whether the two root canals get cleaned using a standard treatment (just small files) or with an experimental treatment (the combination of small files and an ultrasonic dental unit) will be random (by chance, like flipping a coin). My participation or non-participation will have no effect on whether I will receive root canal treatment.

This is done as part of an investigation entitled: **AN EVALUATION OF THE IN VIVO ANTIBACTERIAL EFFICACY OF ULTRASOUND AFTER HAND AND ROTARY INSTRUMENTATION IN HUMAN MANDIBULAR MOLARS**

1. Purpose of the procedure or treatment:

The purpose of this study is to compare how well two different methods of performing root canals used within the mouth remove bacteria.

2. Possible appropriate alternative procedures to treatment (not to participate in the study is always an option):

I may choose not to participate in the study. If I participate, I may withdraw from the study at any time. Not participating will in no way influence the treatment that I will receive in the College of Dentistry. If I am a student and/or employee at OSU, whether or not I choose to participate in this study will have no effect on my grades and/or job.

3. Discomforts and risks reasonably to be expected:

The isopropyl (rubbing) alcohol used to clean my tooth may be irritating to my skin or to my mouth. However, the alcohol will be used in very small quantities, and the risk of any injury (pain or swelling) to me is very remote. There are no other additional risks due to this research over that of having a root canal.

4. Possible benefits for subject/society:

There is no direct health benefit to me if I choose to participate in this study. A better knowledge of how to remove bacteria from root canals is expected from this study. This knowledge may help to prevent the premature loss of teeth.

5. Anticipated duration of the subject's participation, including number of visits, and cost of completing root canal treatment:

I am aware that one appointment is needed to complete the study. The root canal procedure will last approximately 40 minutes, and the bacterial sampling procedure will last approximately 5 minutes. If I am here for emergency root canal treatment (because my tooth hurts), I will have this treatment performed at no charge (a \$90.00 value), and will also receive \$30.00 for my participation. If I am here for regularly scheduled root canal treatment (non-emergency, because my tooth does not hurt), I will receive \$30.00 for participation in this study. One additional follow-up appointment will be required for either emergency or non-emergency cases if I want to have the root canal treatment completed. The total cost for completion of root canal therapy is \$500.00 and is due and payable by me when services are rendered.

I hereby acknowledge that _John Nusstein, D.D.S, M.S.,_ has provided information about the procedure described above, about my rights as a subject, and he/she answered all questions to my satisfaction. I understand that I may contact him/her at phone number 292-5399 should I have additional questions. He/she has explained the risks described above and I understand them; he/she also offered to explain all possible risks or complications.

I understand that, where appropriate, the U.S. Food and Drug Administration may inspect records pertaining to this study. I understand further that records obtained during my participation in this study that may contain my name or other personal

identifiers may be made available to the sponsor of this study. Beyond this, I understand that my participation will remain confidential.

I understand that I am free to withdraw my consent and participation in this project at any time by notifying the project director. No guarantee has been given to me concerning this treatment or procedure.

I understand that in signing this form that, beyond giving consent, I am not waiving any legal rights that I might otherwise have, and I am not releasing the investigator, the sponsor, the institution, or its agents from any legal liability for damages that they might otherwise have.

In the event of injury resulting from participation in this study, I also understand that immediate medical treatment is available at University Hospitals of The Ohio State University and that the costs of such treatment will be at my expense; financial compensation beyond that required by law is not available. Questions about this should be directed to the Office of Responsible Research Practices at 614-688-4792. I have read and fully understand the consent form. I sign it freely and voluntarily. A copy has been given to me.

**Date: _____ Time: ____ AM PM Signed _____
(subject)**

Witness(es) _____

**if Required _____ (Person Authorized to Consent for
Subject if Required)**

I certify that I have personally completed all blanks for this form and explained them to the subject or his/her representative before requiring the subject or his/her representative to sign it.

**Date: _____ Signed: _____
(Signature of Project Director or his/her Authorized Representative)**

APPENDIX D
HIPPA RELEASE FORM

THE OHIO STATE UNIVERSITY
AUTHORIZATION TO USE
PERSONAL HEALTH INFORMATION IN RESEARCH

Title of the Study: An evaluation of the in vivo antibacterial efficacy of ultrasound after hand and rotary instrumentation in human mandibular molars.

OSU Protocol Number: 2004H0116

Principal Investigator: John M. Nusstein, D.D.S., M.S.

Subject Name _____

Before researchers use or share any health information about you as part of this study, The Ohio State University is required to obtain your authorization. This helps explain to you how this information will be used or shared with others involved in the study.

- The Ohio State University and its hospitals, clinics, health-care providers and researchers are required to protect the privacy of your health information.
- You should have received a Notice of Privacy Practices when you received health care services here. If not, let us know and a copy will be given to you. Please carefully review this information. Ask if you have any questions or do not understand any parts of this notice.
- If you agree to take part in this study your health information will be used and shared with others involved in this study. Also, any new health information about you that comes from tests or other parts of this study will be shared with those involved in this study.
- Health information about you that will be used or shared with others involved in this study may include your research record and any health care records at the Ohio State University. For example, this may include your medical records, x-ray or laboratory results. Psychotherapy notes in your health records (if any) will not, however, be shared or used. Use of these notes requires a separate, signed authorization.

Please read the information carefully before signing this form. Please ask if you have any questions about this authorization, the University's Notice of Privacy Practices or the study before signing this form.

Initials/Date: _____

Those Who May Use, Share And Receive Your Information As Part Of This Study

- Researchers and staff at The Ohio State University will use, share and receive your personal health information for this research study. Other Ohio State University staff not involved in the study but who may become involved in your care for study-related treatment will have access to your information.
- Those who oversee the study will have access to your information, including:
 - Members and staff of the Ohio State University's Institutional Review Boards, including the Western Institutional Review Board
 - The Office for Responsible Research Practices
 - University data safety monitoring committees
 - The Ohio State University Research Foundation
- Your health information may also be shared with federal and state agencies that have oversight of the study or to whom access is required under the law. These may include:
 - The Food and Drug Administration
 - The Office for Human Research Protections
 - The National Institutes of Health
 - The Ohio Department of Human Services

These researchers, companies and/or organization(s) outside of The Ohio State University may also use, share and receive your health information in connection with this study:

NONE

The information that is shared with those listed above may no longer be protected by federal privacy rules.

Initials/Date _____

Authorization Period

This authorization will not expire unless you change your mind and revoke it in writing. There is no set date at which your information will be destroyed or no longer used. This is because the information used and created during the study may be analyzed for many years, and it is not possible to know when this will be complete.

Signing the Authorization

- You have the right to refuse to sign this authorization. Your health care outside of the study, payment for your health care, and your health care benefits will not be affected if you choose not to sign this form.
- You will not be able to take part in this study and will not receive any study treatments if you do not sign this form.
- If you sign this authorization, you may change your mind at any time. Researchers may continue to use information collected up until the time that you formally changed your mind. If you change your mind, your authorization must be revoked in writing. To revoke your authorization, please write to:

Dr. John M. Nusstein, 305 W. 12th Avenue, The Ohio State University, Columbus, Ohio 43210

- Signing this authorization also means that you will not be able to see or copy your study-related information until the study is completed. This includes any portion of your medical records that describes study treatment.

Contacts for Questions

- If you have any questions relating to your privacy rights, please contact *Kelly Scheiderer, College of Dentistry and Dental Faculty Practice Privacy Office, 1130 Postle Hall, Columbus, Ohio, 43210*, or by telephone at 614-292-3016.

If you have any questions relating to the research, please contact *Dr. John M. Nusstein, 305 W. 12th Avenue, The Ohio State University, Columbus, Ohio 43210*, or by telephone at 614-292-5399.

Signature

I have read (or someone has read to me) this form and have been able to ask questions. All of my questions about this form have been answered to my satisfaction. By signing below, I permit *Dr. John Nusstein* and the others listed on this form to use and share my personal health information for this study. I will be given a copy of this signed form.

Signature _____
(Subject or Legally Authorized Representative)

Name _____
(Print name above)
(If legal representative, also print relationship to subject.)

Date _____ Time _____ AM / PM

APPENDIX E
BIOMEDICAL SUBMISSION FORMS

BIOMEDICAL SCIENCES

SUMMARY SHEETS

**ADDRESS EACH ITEM IN A COMPLETE AND CONCISE MANNER.
(Do not leave any item blank with "See attached".)**

1. Abstract (overview of research)

The purpose of this prospective, randomized, blinded study is to compare the in vivo antibacterial efficacy of hand and rotary file preparation versus hand and rotary file/ultrasound preparation in the mesial root canals of symptomatic or asymptomatic necrotic mandibular molars.

Eighty subjects with a necrotic symptomatic or asymptomatic posterior mandibular tooth with a minimum 2 x 2 mm mesial periapical radiolucency will be used for this study. The teeth will be divided into two groups. Group 1 will consist of 40 mesial root canals of mandibular molars prepared in vivo (while still in the patients mouth) with a hand and rotary file technique, using intermittent irrigation with 6% sodium hypochlorite, followed by 1 minute of ultrasonic irrigation per canal utilizing an ultrasonic unit set at full power. Group 2 will consist of a parallel control group of 40 mesial root canals of mandibular molars prepared in vivo with a hand and rotary file technique as in group 1 without 1 minute of ultrasonic irrigation. Following standard endodontic access, the tooth and surgical field will be cleaned and disinfected with 70% isopropyl alcohol (Cadco Dental, Oxnard, CA). Liquid dental transport fluid (0.02 ml, Anaerobe systems, Morgan Hill, CA) will be placed in the mesiobuccal and mesiolingual root canals, and the root canals will be sampled for bacteria using sterile paper points prior to instrumentation, following hand and rotary file preparation, and following ultrasonic instrumentation (for teeth in Group 2 only). Cavit[®] will be placed as a temporary restorative material in the endodontic access opening, and the subject will be given an appointment to have the root canal therapy completed or will be referred for extraction of the involved tooth. The samples will be transported in LDT and microbiologically prepared under anaerobic conditions, incubated, and the number of colony forming units (CFUs) per sample will be calculated.

For a non-directional alpha risk of 0.05 and a power of 95%, a sample size of 40 subjects would be required to demonstrate a 25% reduction in pre-ultrasonic bacterial counts. This assumes an initial bacterial count of 5.51×10^7 and a standard deviation of 1.71×10^7 (Shuping GB, Ørstavik D, Sigurdsson A, Trope M. Reduction of intracanal bacteria using nickel-titanium rotary instrumentation and various medications. J Endod 2000;26:751-5). A parallel control group (non-ultrasound) of 40 subjects requires the total population to number 80 subjects.

2. Describe the requirements for a subject population and explain the rationale for using in this population special groups such as prisoners, children, the mentally disabled or groups whose ability to give voluntary informed consent may be in question. Address means of pregnancy screening for females.

The subjects used in this study will be healthy adults who have a necrotic symptomatic or asymptomatic posterior mandibular tooth, which the subject wants treated with endodontic therapy or extraction.

The subjects will be chosen from patients of record and emergency patients of The Ohio State University College of Dentistry who present with a clinical diagnosis of pulpal necrosis with acute or chronic periapical periodontitis with a minimum 2 x 2 mm mesial periapical radiolucency, and are in need of endodontic therapy or extraction. Students and staff may enroll in the study only if they have a posterior mandibular tooth with a clinical diagnosis of pulpal necrosis with acute or chronic periapical periodontitis with a minimum 2 x 2 mm mesial periapical radiolucency and want endodontic therapy or extraction. All subjects will give voluntary informed consent prior to participation, and must fill out a medical history chart. This study will not use any subject who is a prisoner, a child, is mentally retarded, or who is a member of any group whose ability to give voluntary informed consent may be in question. All female subjects will be questioned regarding pregnancy or suspected pregnancy and will not be allowed to participate if pregnant, suspect a pregnancy, nursing, or are trying to become pregnant. Females will be required to take a urine pregnancy test before participation, at the start of the appointment. Patients will not be charged for the urine pregnancy test. The principal investigator will incur the cost for this test.

The precautions to be used to avoid potential coercion or undue influence when enrolling students (or staff) will be as follows: the student will have already made the decision to have endodontic therapy prior to being told about the study; the principal investigator, associates or assistants of his choosing, will not be involved in the initial diagnosis of the tooth or the student's decision to have endodontic therapy.

3. Describe and assess any potential risks - physical, psychological, social, legal, financial, or other - and assess the likelihood and seriousness of such risks. If methods of research create potential risks, describe other methods, if any that were considered and why they will not be used.

A single appointment is needed for this study, per subject, for bacterial sampling. The appointment will consist of an endodontic procedure and bacterial sampling procedure and last approximately 45 minutes. The endodontic portion will take approximately 30-40 minutes, and the bacterial sampling procedure will take approximately 5 minutes.

The risks associated with the bacterial sampling procedure are minimal. The 70% isopropyl alcohol used for field decontamination may cause irritation if allowed to contact skin or during prolonged inhalation of vapors. However, harm only results from

excessive use (MSDS: isopropyl alcohol 70%, Cadco Dental, Oxnard, CA). This product will only be used in minimal quantities and use will be restricted to the operatory field. Therefore, the overall risk to the patient from the use of 70% isopropyl alcohol during this study is minimal. There are no other additional risks due to this research over that of having a root canal.

4. Describe consent procedures to be followed, including how and where informed consent will be obtained. (The use of a finder's fee for recruiting subjects is not permitted.)

The possible risks involved will be explained to the subject's satisfaction prior to the subject reading and signing the consent form.

Prior to the consent procedure, patients will be screened in the Graduate Endodontic clinic or the Emergency clinic at the College of Dentistry. A careful diagnostic procedure will be performed and the patient informed of their treatment options. They will decide what treatment will be performed. They will then be informed of the nature of the current study, and if they fit the criteria they will be referred to the principal investigator. The principal investigator at The Ohio State University College of Dentistry will obtain consent.

5. Describe procedures (including confidentiality safeguards) for protecting against or minimizing potential risks and an assessment of their likely effectiveness.

A six-digit random number will identify subjects and this will be kept strictly confidential to unauthorized personnel. The patient study records will be kept in the principal investigator's, associates' or assistants' of his choosing, office in a locked storage file. Only the principal investigator, associates or assistants of his choosing, will have access to the storage file. Essential emergency medical equipment, drugs, and personnel with CPR and ACLS training will be immediately available, in case of an emergency. The likelihood of a situation arising that can not be handled by said personnel and equipment is extremely remote.

6. Assess the potential benefits to be gained by the individual subject, as well as benefits that may accrue to society in general as a result of the planned work.

There is no direct health benefit to the subject as a result of participating in the study. Subjects presenting for emergency treatment will have this emergency endodontic therapy performed at no charge (a \$90.00 value), and will also receive \$30.00 for their participation. Subjects presenting for routine endodontic therapy will receive \$30.00 for their participation. A better knowledge of endodontic tooth preparation is expected from this study. This knowledge may help to prevent the premature loss of teeth.

7. Compare the risks versus the benefits.

Subjects will be exposed to little risk by participating in this study. The bacterial sampling procedure utilizes no hazardous materials and poses little risk to subjects participating in this study. Because participation in the study will result in very little risk to the subject, the risk-benefit ratio is considered to be favorable.

8. Will the subjects for the study be paid for participating in this study?

☐ No ☒ Yes (\$30.00)

Will subjects be paid for selected activities or for general participation in the study?

Subjects will be paid \$30.00 at the conclusion of the single visit.

Is there any other inducement? If so, please describe.

☐ No ☒ Yes

Emergency endodontic therapy will be provided for all subjects presenting for emergency treatment at no cost (a \$90.00 value). The additional \$30.00 will be paid at the conclusion of the single visit to both emergency subjects and subjects presenting for routine endodontic therapy.

9. Will advertising be used to recruit subjects?

☐ No ☒ Yes - copy attached

Source of funding for proposed research: (Check A or B)

☐ A. OSURF: Sponsor _____ RF Project No. _____

☒ B. Other (identify): Endodontic Support Funds

APPENDIX F
MEDICAL HISTORY FORM

The Ohio State University
College of Dentistry

FORM 1/87

Patient Name _____
Date _____
Date of Birth _____

Medical Inform

Biographical Data _____

Chief Complaint (Why is the patient seeking dental care?) _____

Present Illness (History of Chief Complaint) _____

MEDICAL HISTORY

1. Do you have or have you had any of the following?

a. rheumatic fever or rheumatic heart disease.....	NO	YES
b. heart murmur or mitral valve prolapse.....	NO	YES
c. heart disease/heart attack.....	NO	YES
d. artificial heart valve.....	NO	YES
e. irregular heart beat.....	NO	YES
f. pacemaker.....	NO	YES
g. high blood pressure.....	NO	YES
h. chest pains or angina.....	NO	YES
i. stroke.....	NO	YES
j. artificial joint.....	NO	YES
k. hepatitis/liver disease.....	NO	YES
l. tuberculosis (TB).....	NO	YES
m. thyroid trouble.....	NO	YES
n. kidney disease.....	NO	YES
o. diabetes (sugar).....	NO	YES
p. asthma.....	NO	YES
q. HIV or other immunosuppressive disease.....	NO	YES
r. radiation or cancer therapy.....	NO	YES

2. Do you have or have you had any disease, condition, or problem not listed here? NO YES

3. Have you ever been hospitalized? NO YES

4. Have you had excessive or prolonged bleeding requiring special treatment? NO YES

5. Have you had an allergic reaction to any drugs or medications?
(Circle all that apply: penicillin; codeine; aspirin; anesthetics; other) NO YES

6. Are you currently under the care of a physician (M.D., D.O.)? NO YES

When were you last seen by a physician? _____
Name of Physician _____
Street address _____
City, State, and Zip Code _____
Phone _____

7. Are you pregnant or nursing? Estimated Date of Delivery _____ NO YES

8. Have you had any trouble associated with previous dental treatment? NO YES

9. Do you have any lumps or sores in your mouth now? NO YES

10. Do you smoke or use smokeless tobacco? NO YES

11. How often do you have dental check ups? _____ Date of last Exam _____

12. Are you currently taking any drugs or medications
(such as antibiotics, heart medicine, birth control pills?)..... NO YES

CURRENT MEDICATIONS

Trade Name	Generic Name	Dose/Frequency	Reason

I have reviewed the information I have provided, and to the best of my knowledge it is correct and complete.

Patient/Guardian Signature _____ Date: _____

SUMMARY OF PATIENT'S MEDICAL STATUS: _____

MEDICAL RISK ASSESSMENT (check one)

- ☐ ASA I (healthy individual) ☐ ASA III (severe disease but not incapacitating)
☐ ASA II (mild systemic disease) ☐ ASA IV (incapacitating systemic disease, constant threat to life)

MEDICAL CONSULTATION REQUIRED

- ☐ No (healthy and/or stabilized disease)
☐ Yes (ASA III or IV; cardiac murmur; vague hx; recent major disease; recent diagnosis/operation; uncontrolled disease; blood pressure; etc.)

Does the chief complaint require emergency treatment?..... NO YES

Student / I.D.#

Instructor / I.D.#

Date

APPENDIX G
MASTER CODE SHEET

Subject Number	ID Number	Group
1	677621	1
2	641165	1
3	298690	2
4	663078	1
5	946182	2
6	550273	2
7	473866	1
8	766751	2
9	303692	1
10	442734	2
11	256803	2
12	245476	2
13	627638	2
14	328726	2
15	967021	2
16	525584	1
17	920203	1
18	816690	1
19	658996	1
20	470304	1
21	860122	1
22	281809	1
23	468391	2
24	135858	1
25	444244	1
26	499158	2
27	947183	2
28	404311	1
29	957769	2
30	737082	2
31	453062	2
32	348869	2
33	497112	2
34	578483	1
35	408041	1
36	865207	2
37	170518	2
38	644737	1
39	357820	1
40	925186	1

APPENDIX H

RAW DATA

Sub #	Age (yrs)	Sex 0=Fem 1=Male	Tooth 0=1 st molar 1=2 nd molar	PA Lesion Area (mm ²)	Canal Type 1=I 2=II 3=III 4=IV 5=C	Curv. (Deg.)	Method 0=No Ultra 1=Ultra	Working Length (mm)
281809	34	1	1	36	3	40	0	21
947183	38	0	1	25	2	8	1	22
860122	21	1	1	44	3	10	0	20
135858	31	1	2	64	2	7	0	24
470304	44	0	1	25	2	8	0	22
256803	26	1	1	53	2	29	1	19
658996	25	0	1	12	2	16	0	23
303692	52	0	2	9	3	19	0	18
442734	28	1	1	6	3	8	1	21
245476	66	1	2	54	2	61	1	21
468391	42	1	1	5	3	29	1	22
663078	54	1	2	42	5	14	0	21
473866	55	0	1	63	3	25	0	21
298690	20	1	1	36	3	16	1	20
677621	26	1	2	48	2	56	0	22
641165	42	1	2	32	2	30	0	23
737082	36	1	1	40	2	7	1	23
957769	56	0	2	90	3	18	1	19
816690	21	1	1	132	2	29	0	21
404311	20	1	1	9	3	16	0	20
499158	26	1	1	77	2	31	1	21
946182	51	0	2	40	1	20	1	17
328726	46	1	1	14	3	22	1	23
444244	37	0	1	12	3	3	0	18
550273	45	0	1	20	3	12	1	18
967021	64	0	1	54	2	30	1	19
525584	33	1	1	24	3	6	0	20
627638	45	1	1	16	2	25	1	23
920203	64	0	1	16	2	27	0	18
766751	48	1	1	28	3	32	1	22
578483	30	0	2	6	3	11	0	20
453062	19	1	2	24	2	19	1	20
408041	22	1	1	6	2	23	0	22
663078	54	1	2	42	5	14	0	21
946182	51	0	2	40	1	20	1	17

Sub #	Ultra Needle Penetration (mm)	Final Taper 1=.04 2=.06	S1	S2	S3	S1 PCR 0=neg 1=pos	S2 PCR 0=neg 1=pos	S3 PCR 0=neg 1=pos
281809	.	1	5000000	0	.	.	.	.
947183	11	1	60000	40	0	.	.	.
860122	.	2	640000	80	.	.	.	.
135858	.	1	3000	0	.	.	.	.
470304	.	1	84000	0	.	.	.	.
256803	9	1	376000	0	0	.	.	.
658996	.	2	200000	160	.	.	.	.
303692	.	1	240	40	.	.	.	.
442734	15	2	1000000	0	0	.	.	.
245476	14	1	28400	0	0	.	.	.
468391	12	1	2320000	2360	600	.	.	.
663078	.	1	1160000	3440	.	1	0	.
473866	.	2	64800	0	.	.	.	.
298690	13	2	720000	240	80	1	1	0
677621	.	1	1720000	0	.	.	.	.
641165	.	1	36400	120	.	1	0	.
737082	13	2	2680000	2960	1520	1	1	1
957769	13	2	356000	360	0	0	0	.
816690	.	1	172000	0	.	.	.	.
404311	.	1	3080000	80	.	1	1	.
499158	15	1	880000	1640	0	1	1	.
946182	12	2	6800	0	0	.	.	.
328726	15	1	30000	0	0	.	.	.
444244	.	2	5440000	180000	.	0	0	.
550273	14	2	236000	16000	0	1	1	.
967021	12	2	604000	4000	0	.	.	.
525584	.	2	48000	13600	2400	.	.	.
627638	15	1	192000	300	0	.	.	.
920203	.	2	80000	60	40	.	.	.
766751	15	2	2560000	88000	0	.	.	.
578483	.	2	480000	0	0	.	.	.
453062	15	2	4080000	44000	0	.	.	.
408041	.	1	88000	600	400	.	.	.
663078	.	1	1160000	3440	.	1	0	.
946182	12	2	6800	0	0	.	.	.

LIST OF REFERENCES

1. Schilder H. Cleaning and shaping of the root canal. *Dent Clin North Am* 1974;18:269-96.
2. West JD, Roane JB. Cleaning and shaping the root canal system. In: Cohen S, Burns RC. *Pathways of the Pulp*, 7th ed. St. Louis: CV Mosby Co., 1998:203-57.
3. Weine FS. *Endodontic Therapy*, 5th ed. St. Louis: CV Mosby Co., 1996:239-304.
4. Sjögren U, Sundqvist G, Nair PNR. Factors affecting the long-term results of endodontic treatment. *J Endod* 1990;16:498-504.
5. Pineda F, Kuttler Y. Mesiodistal and buccolingual roentgenographic investigation of 7,275 root canals. *Oral Surg* 1972;33:101-10.
6. Green D. Stereomicroscopic study of 700 root apices of maxillary and mandibular posterior teeth. *Oral Surg* 1960;13:728-33.
7. Kuttler Y. Microscopic investigation of root apices. *J Am Dent Assoc* 1950;50:544-52.
8. Green EN. Microscopic investigation of root canal diameters. *J Am Dent Assoc* 1958;57:636-44.
9. Skidmore AE, Bjorndal AM. Root canal morphology of the human mandibular first molar. *Oral Surg* 1971;32:778-84.
10. Hess W. *The Anatomy of the Root-Canals of the Teeth of the Permanent Dentition-Part I*, London: John Bale, Sons, and Danielsson, 1925:3-49.
11. Vertucci FJ. Root canal anatomy of the human permanent teeth. *Oral Surg* 1984;58:589-99.
12. Manning SA. Root canal anatomy of mandibular second molars. Part I. *Int Endod J* 1990;23:34-9.

13. Yesilsoy C, Gordon W, Porras O, Hoch B. Observation of depth and incidence of the mesial groove between the mesiobuccal and mesiolingual orifices in mandibular molars. *J Endod* 2002;28:507-9.
14. Weller RN, Brady JM, Bernier WE. Efficacy of ultrasonic cleaning. *J Endod* 1980;6:740-3.
15. Goodman A, Reader A, Beck F, Melfi R, Meyers W. An in vitro comparison of the efficacy of the step-back technique versus a step-back ultrasonic technique in human mandibular molars. *J Endod* 1985;11:249-56.
16. Lev R, Reader A, Beck M, Meyers W. An in vitro comparison of the step-back technique versus a step-back/ultrasound technique for one and three minutes. *J Endod* 1987;13:523-30.
17. Haidet J, Reader A, Beck M, Meyers W. An in vivo comparison of the step-back technique versus a step-back/ultrasonic technique in human mandibular molars. *J Endod* 1989;15:195-9.
18. Metzler RS, Montgomery S. The effectiveness of ultrasonics and calcium hydroxide for the debridement of human mandibular molars. *J Endod* 1989;15:373-8.
19. Jensen SA, Walker TL, Hutter JW, Nicoll BK. Comparison of the cleaning efficacy of passive sonic activation and passive ultrasonic activation after hand instrumentation in molar root canals. *J Endod* 1999;25:735-8.
20. Mayer BE, Peters OA, Barbakow F. Effects of rotary instruments and ultrasonic irrigation on debris and smear layer scores: a scanning electron microscopic study. *Int Endod J* 2002;35:582-9.
21. Cameron JA. The choice of irrigant during hand instrumentation and ultrasonic irrigation of the root canal: a scanning electron microscope study. *Aust Dent J* 1995;40:85-90.
22. Schafer E, Zapke K. A comparative scanning electron microscopic investigation of the efficacy of manual and automated instrumentation of root canals. *J Endod* 2000;26:660-4.
23. Hulsmann M, Schade M, Schafers F. A comparative study of root canal preparation with HERO 642[®] and Quantec[®] SC rotary Ni-Ti instruments. *Int Endod J* 2001;34:538-46.

24. Versumer J, Hulsmann M, Schafers F. A comparative study of root canal preparation using ProFile[®].04 and LightSpeed[®] rotary Ni-Ti instruments. *Int Endod J* 2002;35:37-46.
25. Mayer BE, Peters OA, Barbakow F. Effects of rotary instruments and ultrasonic irrigation on debris and smear layer scores: a scanning electron microscopic study. *Int Endod J* 2002;35:582-9.
26. Tan BT, Messer HH. The quality of apical canal preparation using hand and rotary instruments with specific criteria for enlargement based on initial apical file size. *J Endod* 2002;28:658-64.
27. Archer R, Reader A, Nist R, Beck M, Meyers WJ. An in vivo evaluation of the efficacy of ultrasound after step-back preparation in mandibular molars. *J Endod* 1992;18:549-52.
28. Byström A, Sundqvist G. Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. *Scand J Dent Res* 1981;89:321-8.
29. Dalton BC, Ørstavik D, Phillips C, Pettiette M, Trope M. Bacterial reduction with nickel-titanium rotary instrumentation. *J Endod* 1998;24:763-7.
30. Shuping GB, Ørstavik D, Sigurdsson A, Trope M. Reduction of intracanal bacteria using nickel-titanium rotary instrumentation and various medications. *J Endod* 2000;26:751-5.
31. Card SJ, Sigurdsson A, Ørstavik D, Trope M. The effectiveness of increased apical enlargement in reducing intracanal bacteria. *J Endod* 2002;28:779-83.
32. Martin H. Ultrasonic disinfection of the root canal. *Oral Surg* 1976;42:92-9.
33. Martin H, Cunningham WT, Norris JP, Cotton WR. Ultrasonic versus hand filing of dentin: a quantitative study. *Oral Surg* 1980;49:79-81.
34. Martin H, Cunningham, WT, Norris, JP. A quantitative comparison of the ability of diamond and K-type files to remove dentin. *Oral Surg* 1980;50:566-8.
35. Cunningham WT, Martin H, Forrest WR. Evaluation of root canal debridement by the Endosonic ultrasonic synergistic system. *Oral Surg* 1982;53:401-4.
36. Cunningham WT, Martin H. A scanning electron microscope evaluation of root canal debridement with the Endosonic ultrasonic synergistic system. *Oral Surg* 1982;53:527-31.

37. Martin H, Cunningham WT. The effect of Endosonic and hand manipulation on the amount of root canal material extruded. *Oral Surg* 1982;53:611-3.
38. Martin H, Cunningham WT. An evaluation of postoperative pain incidence following Endosonic and conventional root canal therapy. *Oral Surg* 1982;54:74-6.
39. Tauber R, Morse D, Sinai I, Furst M. A magnifying lens comparative evaluation of conventional and ultrasonically energized filing. *J Endod* 1983;9:269-74.
40. Cymerman J, Jerome L, Moodnik R. A scanning electron microscope study comparing the efficacy of hand instrumentation with ultrasonic instrumentation of the root canal. *J Endod* 1983;9:327-31.
41. Langeland K, Liao K, Pascon E. Work saving devices in endodontics: efficacy of sonic and ultrasonic techniques. *J Endod* 1985;11:499-510.
42. Pedicord D, ElDeeb M, Messer H. Hand versus ultrasonic instrumentation: its effect on canal shape and instrumentation time. *J Endod* 1986;12:375-80.
43. Reynolds M, Madison S, Walton R, Krell K, Rittman B. An in vitro histological comparison of the step-back, sonic, and ultrasonic instrumentation techniques in small, curved root canals. *J Endod* 1987;13:307-14.
44. Goldman M, White R, Moser C, Tenca, J. A comparison of three methods of cleaning and shaping the root canal in vitro. *J Endod* 1988;14:7-12.
45. Ahmad M, PittFord TR, Crum LA. Ultrasonic debridement of root canals: an insight into the mechanisms involved. *J Endod* 1987;13:93-101.
46. Ahmad M, PittFord TR, Crum LA. Ultrasonic debridement of root canals: acoustic streaming and its possible role. *J Endod* 1987;13:490-9.
47. Ahmad M, PittFord, TR, Crum LA. Ultrasonic debridement of root canals: acoustic cavitation and its relevance. *J Endod* 1988;14:486-93.
48. Ahmad M. The validity of using simulated root canals as models for ultrasonic instrumentation. *J Endod* 1989;15:544-7.
49. Walmsley AD. Ultrasound and root canal treatment: the need for scientific evaluation. *Int Endod J* 1987;20:105-11.
50. Graff KF. Process applications of power ultrasonics-a review. *IEEE Symposium on Sonics and Ultrasonics, Milwaukee, Wisconsin, 1974:1-14.*

51. Stamos D, Sadeghi E, Haasch G, Gerstein H. An in vitro comparison study to quantitate the debridement ability of hand, sonic, and ultrasonic instrumentation. J Endod 1987;13:434-40.
52. Roy RA, Ahmad M, Crum LA. Physical mechanisms governing the hydrodynamic response of an oscillating ultrasonic file. Int Endod J 1994;27:197-207.
53. Siqueira JF, Araujo MC, Garcia PF, Fraga RC, Dantas CJ. Histological evaluation of the effectiveness of five instrumentation techniques for cleaning the apical third of root canals. J Endod 1997;23:499-502.
54. Heard F, Walton RE. Scanning electron microscope study comparing four root canal preparation techniques in small curved canals. Int Endod J 1997;30:323-31.
55. Sjögren U, Sundqvist G. Bacteriologic evaluation of ultrasonic root canal instrumentation. Oral Surg Oral Med Oral Pathol 1987;63:366-70.
56. Yoshida T, Shibata T, Shinohara T, Gomyo S, Sekine I. Clinical evaluation of the efficacy of EDTA solution as an endodontic irrigant. J Endod 1995;21:592-3.
57. Spoleti P, Siragusa M, Spoleti MJ. Bacteriological evaluation of passive ultrasonic activation. J Endod 2003;29:12-4.
58. Ahmad M, Pitt Ford TR, Crum LA, Wilson RF. Effectiveness of ultrasonic files in the disruption of root canal bacteria. Oral Surg Oral Med Oral Pathol 1990;70:328-32.
59. Huque J, Kota K, Yamaga M, Iwaku M, Hoshino E. Bacterial eradication from root dentine by ultrasonic irrigation with sodium hypochlorite. Int Endod J 1998;31:242-50.
60. Siqueira JF, Machado AG, Silveira RM, Lopes HP, de Uzeda M. Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal, in vitro. Int Endod J 1997;30:279-82.
61. Jensen SA, Walker TL, Hutter JW, Nicoll BK. Comparison of the cleaning efficacy of passive sonic activation and passive ultrasonic activation after hand instrumentation in molar root canals. J Endod 1999;25:735-8.

62. Gutarts RR, Nusstein J, Reader A, Beck FM. An evaluation of the in vivo debridement efficacy of ultrasound after hand and rotary instrumentation in human mandibular molars. Master's Thesis. The Ohio State University, 2003.
63. Miller W. Introduction in the study of the bacteriopathology of the dental pulp. *Dent Cosmos* 1894;36:505.
64. Kakehashi S, Stanley HR, Fitzgerald RJ. The effect of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surg Oral Med Oral Pathol* 1965;20:340-9.
65. Möller ÅJR, Fabricius L, Dahlén G, Heyden G. Influence on periradicular tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. *Scand J Dent Res* 1981;89:475-84.
66. Fabricius L, Dahlén G, Holm SE, Möller ÅJR. Influence of combinations of oral bacteria on periapical tissues of monkeys. *Scand J Dent Res* 1982;90:200-6.
67. Gomes BP, Drucker DB, Lilley JD. Positive and negative associations between bacterial species in dental root canals. *Microbios* 1994;80:231-43.
68. Sundqvist G. Associations between microbial species in dental root canal infections. *Oral Microbiol Immunol* 1992;7:257-62.
69. Grenier D, Mayrand D. Nutritional relationships between oral bacteria. *Infect Immun* 1986;53:616-20.
70. Riley MA, Wertz JE. Bacteriocins: evolution, ecology, and application. *Annu Rev Microbiol* 2002;56:117-37.
71. Kobayashi T, Hayashi A, Yoshikawa R, Okuda, K, Hara K. The microbial flora from root canals and periodontal pockets of non-vital teeth associated with advanced periodontitis. *Int Endod J* 1990;23:100-6.
72. Fabricius L, Dahlén G, Öhman AE, Möller ÅJR. Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure. *Scand J Dent Res* 1982;90:134-44.
73. Baumgartner JC, Falkler WA. Bacteria in the apical 5 mm of infected root canals. *J Endod* 1991;17:380-3.
74. Sundqvist G. Taxonomy, ecology, and pathogenicity of the root canal flora. *Oral Surg Oral Med Oral Pathol* 1994;78:522-30.

75. Kantz WE, Henry CA. Isolation and classification of anaerobic bacteria from intact pulp chambers of non-vital teeth in man. *Arch Oral Biol* 1974;19:91-6.
76. Wittgow WC, Sabiston CB. Microorganisms from pulpal chambers of intact teeth with necrotic pulps. *J Endod* 1975;1:168-71.
77. Drucker DB, Lilley JD, Tucker D, Gibbs AC. The endodontic microflora revisited. *Microbios* 1992;71:225-34.
78. Bae KS, Baumgartner JC, Shearer TR, David LL. Occurrence of *Prevotella nigrescens* and *Prevotella intermedia* in infections of endodontic origin. *J Endod* 1997;23:620-3.
79. Gharbia SE, Haapasalo, M, Shah HN, Kotiranta A, Lounatmaa K, Pearce MA, Devine DA. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. *J Periodontol* 1994;65:56-61.
80. Dougherty WJ, Bae KS, Watkins BJ, Baumgartner JC. Black-pigmented bacteria in coronal and apical segments of infected root canals. *J Endod* 1998;24:356-8.
81. Xia, T, Baumgartner JC, David LL. Isolation and identification of *Prevotella tanneriae* from endodontic infections. *Oral Microbiol Immunol* 2000;15:273-5.
82. Machado de Oliveira JC, Siqueira JF, Alves GB, Hirata R, Andrade AF. Detection of *Porphyromonas endodontalis* in infected root canals by 16S rRNA gene-directed polymerase chain reaction. *J Endod* 2000;26:729-32.
83. Van Winkelhoff AJ, van Steenberghe TJ, de Graaff, J. *Porphyromonas (Bacteroides) endodontalis*: its role in endodontal infections. *J Endod* 1992;18:431-4.
84. Kilian M. Degradation of immunoglobulins A1, A2, and G by suspected principal periodontal pathogens. *Infect Immun* 1981;34:757-65.
85. Siqueira JF, Rôças IN, Souto R, de Uzeda M, Colombo AP. Checkerboard DNA-DNA hybridization analysis of endodontic infections. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000;89:744-8.
86. Baumgartner JC, Falkler WA, Beckerman T. Experimentally induced infection by oral anaerobic microorganisms in a mouse model. *Oral Microbiol Immunol* 1992;7:253-6.

87. Price SB, McCallum RE. Studies on bacterial synergism in mice infected with *Bacteroides intermedius* and *Fusobacterium necrophorum*. J Basic Microbiol 1987;27:377-86.
88. Sundqvist GK, Eckerbom MI, Larsson AP, Sjögren UT. Capacity of anaerobic bacteria from necrotic dental pulps to induce purulent infections. Infect Immunol 1979;25:685-93.
89. Iwu C, MacFarlane TW, MacKenzie D, Stenhouse D. The microbiology of periapical granulomas. Oral Surg Oral Med Oral Pathol 1990;69:502-5.
90. Abou-Rass M, Bogen G. Microorganisms in closed periapical lesions. Int Endod J 1998;31:39-47.
91. Wayman BE, Murata SM, Almeida RJ, Fowler CB. A bacteriological and histological evaluation of 58 periapical lesions. J Endod 1992;18:152-5.
92. Gatti JJ, Dobeck JM, Smith C, White RR, Socransky SS, Skobe Z. Bacteria of asymptomatic periradicular endodontic lesions identified by DNA-DNA hybridization. Endod Dent Traumatol 2000;16:197-204.
93. Walton RE, Ardjmand K. Histological evaluation of the presence of bacteria in induced periapical lesions in monkeys. J Endod 1992;18:216-21.
94. Andreason I, Rud J. A histobacteriologic study of dental and periapical structures after endodontic surgery. Int J Oral Surg 1972;1:272-81.
95. Block RM, Bushell A, Rodrigues H, Langeland K. A histopathologic, histobacteriologic, and radiographic study of periapical endodontic surgical specimens. Oral Surg 1976;42:656-78.
96. Langeland K, Block R, Grossman L. A histopathological and histobacteriological study of 35 periapical endodontic surgical specimens. J Endod 1977;3:8-23.
97. Sabeti M, Slots J. Herpesviral-bacterial coinfection in periapical pathosis. J Endod 2004;30:69-72.
98. Baumgartner JC, Watts CM, Xia T. Occurrence of *Candida albicans* in infections of endodontic origin. J Endod 2000;26:695-8.
99. Sen BH, Piskin B, Demirci T. Observation of bacteria and fungi in infected root canals and dentinal tubules by SEM. Endod Dent Traumatol 1995;11:6-9.

100. Siqueira JF, Rôças IN, Lopes HP. Patterns of microbial colonization in primary root canal infections. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;93:174-8.
101. Waltimo TM, Sirén EK, Torkko HL, Olsen I, Haapasalo MP. Fungi in therapy-resistant apical periodontitis. *Int Endod J* 1997;30:96-101.
102. Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1998;85:86-93.
103. Molander A, Reit C, Dahlén G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. *Int Endod J* 1998;31:1-7.
104. Baumgartner JC, Khemaleelakul S, Xia T. Identification of spirochetes (treponemes) in endodontic infections. *J Endod* 2003;29:794-7.
105. Siqueira JF, Rôças IN, Favieri A, Oliveira JC, Santos KR. Polymerase chain reaction detection of *Treponema denticola* in endodontic infections within root canals. *Int Endod J* 2001;34:280-4.
106. Siqueira JF, Rôças IN. *Treponema socranskii* in primary endodontic infections as detected by nested PCR. *J Endod* 2003;29:244-7.
107. Sundqvist G, Johansson E, Sjögren U. Prevalence of black-pigmented *Bacteroides* species in root canal infections. *J Endod* 1989;15:13-9.
108. Van Winkelhoff AJ, Carlee AW, de Graaff J. *Bacteroides endodontalis* and other black-pigmented *Bacteroides* species in odontogenic abscesses. *Infect Immun* 1985;49:494-7.
109. Griffie MB, Patterson SS, Miller CH, Kafrawy AH, Newton CW. The relationship of *Bacteroides melaninogenicus* to symptoms associated with pulpal necrosis. *Oral Surg Oral Med Oral Pathol* 1980;50:457-61.
110. Heimdahl A, von Konow L, Satoh T, Nord CE. Clinical appearance of orofacial infections of odontogenic origin in relation to microbiological findings. *J Clin Microbiol* 1985;22:299-302.
111. Hashioka K, Yamasaki M, Nakane A, Horiba N, Nakamura H. The relationship between clinical symptoms and anaerobic bacteria from infected root canals. *J Endod* 1992;18:558-61.
112. Gomes BP, Lilley JD, Drucker DB. Clinical significance of dental root canal microflora. *J Dent* 1996;24:47-55.

113. Haapasalo M, Ranta H, Ranta K, Shah H. Black-pigmented *Bacteroides* spp. in human apical periodontitis. *Infect Immun* 1986;53:149-53.
114. Haapasalo M. *Bacteroides* spp. in dental root canal infections. *Endod Dent Traumatol* 1989;5:1-10.
115. Baumgartner JC, Watkins BJ, Bae KS, Xia T. Association of black-pigmented bacteria with endodontic infections. *J Endod* 1999;25:413-5.
116. Siqueira JF, Rôças IN. *Bacteroides forsythus* in primary endodontic infections as detected by nested PCR. *J Endod* 2003;29:390-3.
117. Sjögren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *Int Endod J* 1997;30:297-306.
118. Nair PN, Sjögren U, Krey G, Kahnberg KE, Sundqvist G. Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. *J Endod* 1990;16:580-7.
119. Hoshino E, Ando N, Sato M, Kota K. Bacterial invasion of non-exposed dental pulp. *Int Endod J* 1992;25:2-5.
120. Love RM. Regional variation in root dentinal tubule infection by *Streptococcus gordonii*. *J Endod* 1996;22:290-3.
121. Nagaoka S, Miyazaki Y, Liu HJ, Iwamoto Y, Kitano M, Kawagoe M. Bacterial invasion into dentinal tubules of human vital and nonvital teeth. *J Endod* 1995;21:70-3.
122. Drake DR, Wiemann AH, Rivera EM, Walton RE. Bacterial retention in canal walls in vitro: effect of smear layer. *J Endod* 1994;20:78-82.
123. Perez F, Calas P, Rochd T. Effect of dentin treatment on in vitro root tubule bacterial invasion. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1996;82:446-51.
124. Byström A, Sundqvist G. Bacteriologic evaluation of the effect of 0.5 percent sodium hypochlorite in endodontic therapy. *Oral Surg* 1983;55:307-12.
125. Byström A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. *Int Endod J* 1985;18:35-40.

126. Byström A, Claesson R, Sundqvist G. The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. *Endod Dent Traumatol* 1985;1:170-5.
127. Byström A, Happonen RP, Sjögren U, Sundqvist G. Healing of periapical lesions of pulpless teeth after endodontic treatment with controlled asepsis. *Endod Dent Traumatol* 1987;3:58-63.
128. Kerekes K, Tronstad L. Morphologic observations on root canals of human molars. *J Endod* 1977;3:114-8.
129. Ørstavik D, Kerekes K, Molven O. Effects of extensive apical reaming and calcium hydroxide dressing on bacterial infection during treatment of apical periodontitis: a pilot study. *Int Endod J* 1991;24:1-7.
130. Coldero LG, McHugh S, MacKenzie D, Saunders WP. Reduction in intracanal bacteria during root canal preparation with and without apical enlargement. *Int Endod J* 2002;35:437-46.
131. Rollison S, Barnett F, Stevens RH. Efficacy of bacterial removal from instrumented root canals in vitro related to instrumentation technique and size. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;94:366-71.
132. Siqueira JF, Lima KC, Magalhaes FA, Lopes HP, de Uzeda M. Mechanical reduction of the bacterial population in the root canal by three instrumentation techniques. *J Endod* 1999;25:232-5.
133. Siqueira JF, Rôças IN, Favieri A, Lima KC. Chemomechanical reduction of the bacterial population in the root canal after instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite. *J Endod* 2000;26:331-4.
134. Siqueira JF, Rôças IN, Santos SRLD, Lima KC, Magalhaes FAC, de Uzeda M. Efficacy of instrumentation techniques and irrigation regimens in reducing the bacterial population within root canals. *J Endod* 2002;28:181-4.
135. Pataky L, Ivanyi I, Grigar A, Fazekas A. Antimicrobial efficacy of various root canal preparation techniques: an in vitro comparative study. *J Endod* 2002;28:603-5.
136. Peters LB, van Winkelhoff AJ, Buijs JF, Wesselink PR. Effects of instrumentation, irrigation and dressing with calcium hydroxide on infection in pulpless teeth with periapical bone lesions. *Int Endod J* 2002;35:13-21.

137. Piccolomini R, D'Arcangelo C, D'Ercole S, Catamo G, Schiaffino G, De Fazio P. Bacteriologic evaluation of the effect of Nd:YAG laser irradiation in experimental infected root canals. *J Endod* 2002;28:276-8.
138. Barnett F, Trope M, Khoja M, Tronstad L. Bacteriologic status of the root canal after sonic, ultrasonic, and hand instrumentation. *Endod Dent Traumatol* 1985;1:228-31.
139. DeNunzio MS, Hicks ML, Pelleu GB, Kingman A, Sauber JJ. A bacteriological comparison of ultrasonic and hand instrumentation of root canals in dogs. *J Endod* 1989;14:290-3.
140. Ahmad M. Effect of ultrasonic instrumentation on *Bacteroides intermedius*. *Endod Dent Traumatol* 1989;5:83-6.
141. Fegan SE, Steiman HR. Comparative evaluation of the antibacterial effects of intracanal Nd:YAG laser irradiation: an in vitro study. *J Endod* 1995;21:415-7.
142. Weber CD, McClanahan SB, Miller GA, Diener-West, M, Johnson JD. The effect of passive ultrasonic activation of 2% chlorhexidine or 5.25% sodium hypochlorite irrigant on residual antimicrobial activity in root canals. *J Endod* 2003;29:562-4.
143. Ruoff KL, de la Maza L, Murtagh MJ, Spargo JD, Ferraro MJ. Species identities of enterococci isolated from clinical specimens. *J Clin Microbiol* 1990;28:435-7.
144. Lewis CM, Zervos MJ. Clinical manifestations of enterococcal infection. *Eur J Clin Microbiol Infect Dis* 1990;9:111-7.
145. Murray BE. The life and times of the *Enterococcus*. *Clin Microbiol Rev* 1990;3:46-65.
146. Moellering RC. The Garrod Lecture. The *Enterococcus*: a classic example of the impact of antimicrobial resistance on therapeutic options. *J Antimicrob Chemother* 1991;28:1-12.
147. Schaberg DR, Culver DH, Gaynes RP. Major trends in the microbial etiology of nosocomial infection. *Am J Med* 1991;91:72-5.
148. Prescott LM, Harley JP, Klein DA. Microbiology, 3rd ed. Chicago Wm. C. Brown Publishers, 1996:443-44.

149. Kreft B, Marre R, Schramm U, Wirth R. Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. *Infect Immun* 1992;60:25-30.
150. Shankar B, Baghdayan AS, Huycke MM, Lindahl G, Gillmore MS. Infection derived *Enterococcus faecalis* strains are enriched in esp, a gene encoding a novel surface protein. *Infect Immun* 1999;67:193-200.
151. Hase CC, Finkelstein RA. Bacterial extracellular zinc containing metalloproteinases. *Microbiol Rev* 1993;57:823-37.
152. Miyazaki S, Ohno A, Kobayashi I, Uji T, Yamaguchi I, Goto S. Cytotoxic effect of hemolytic culture supernatant from *Enterococcus faecalis* on mouse polymorphonuclear neutrophils and macrophages. *Microbiol Immunol* 1993;37:265-70.
153. Mundy LM, Sahm DF, Gilmore M. Relationships between enterococcal virulence and antimicrobial resistance. *Clin Microbiol Rev* 2000;13:513-22.
154. Oguntebi B, Slee AM, Tanzer JM, Langeland K. Predominant microflora associated with human dental periapical abscesses. *J Clin Microbiol* 1982;15:964-6.
155. De Sousa EL, Ferraz CC, Gomes BP, Pinheiro ET, Teixeira FB, Souza-Filho FJ. Bacteriological study of root canals associated with periapical abscesses. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2003;96:332-9.
156. Engström B. The significance of enterococci in root canal treatment. *Odontol Revy* 1964;15:87-106.
157. Siqueira JF, Rôças IN, Souto R, de Uzeda M, Colombo AP. *Actinomyces* species, streptococci, and *Enterococcus faecalis* in primary root canal infections. *J Endod* 2002;28:168-72.
158. Mejare B. *Streptococcus faecalis* and *Streptococcus faecium* in infected dental root canals at filling and their susceptibility to azidocillin and some comparable antibiotics. *Odontol Revy* 1975;26:193-204.
159. Sirén EK, Haapasalo MP, Ranta K, Salmi P, Kerosuo EN. Microbiological findings and clinical treatment procedures in endodontic cases selected for microbiological investigation. *Int Endod J* 1997;30:91-5.
160. Pinheiro ET, Gomes BP, Ferraz CC, Sousa EL, Teixeira FB, Souza-Filho FJ. Microorganisms from canals of root-filled teeth with periapical lesions. *Int Endod J* 2003;36:1-11.

161. Waltimo T, Sirén E, Ørstavik D, Haapasalo M. Susceptibility of oral *Candida* species to calcium hydroxide in vitro. *Int Endod J* 1999;32:94-8.
162. Ørstavik D, Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endod Dent Traumatol* 1990;6:142-9.
163. Gomes BPFA, Ferraz CCR, Vianna ME, Berber VB, Teixeira FB, Souza-Filho FJ. In vitro antimicrobial activity of several concentrations of sodium hypochlorite, and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. *Int Endod J* 2001;34:424-8.
164. Evanov C, Liewehr F, Buxton TB, Joyce AP. Antibacterial efficacy of calcium hydroxide and chlorhexidine gluconate irrigants at 37°C and 46°C. *J Endod* 2004;30:653-7.
165. Schäfer E, Bössmann K. Antimicrobial efficacy of chlorhexidine and two calcium hydroxide formulations against *Enterococcus faecalis*. *J Endod* 2005;31:53-6.
166. Cwikla SJ, Bélanger M, Giguère S, Progulske-Fox A, Vertucci FJ. Dentinal tubule disinfection using three calcium hydroxide formulations. *J Endod* 2005;31:50-2.
167. Peciuliene V, Reynaud A, Balciuniene I, Haapasalo M. Isolation of yeasts and enteric bacteria in root-filled teeth with chronic apical periodontitis. *Int Endod J* 2001;34:429-34.
168. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. *Int Endod J* 2002;35:221-8.
169. Figdor D, Davies JK, Sundqvist G. Starvation survival, growth and recovery of *Enterococcus faecalis* in human serum. *Oral Microbiol Immunol* 2003;18:234-9.
170. Shabahang S, Torabinejad M. Effect of MTAD™ on *Enterococcus faecalis*-contaminated root canals of extracted human teeth. *J Endod* 2003;29:576-9.
171. Zielke DR, Heggers JP, Harrison JW. An analysis of the sensitivity of non-reduced PRS medium in endodontic therapy. *Oral Surg Oral Med Oral Pathol* 1979;47:83-6.

172. Griffie MB, Patterson SS, Miller CH, Kafrawy AH, Newton CW. Comparison of bacterial growth in an improperly but commonly used medium versus reduced thioglycollate with the use of an anaerobic sampling technique. *Oral Surg Oral Med Oral Pathol* 1981;52:433-6.
173. Palmer GR, Lazzarotto JR, Weine FS. Paper point transfer of minimal numbers of microorganisms from a prepared canal in vitro. *Oral Surg Oral Med Oral Pathol* 1976;42:824-9.
174. Marshall FJ, Savoie FL. Efficiency of endodontic culturing procedures using wet and dry paper points. *Oral Surg Oral Med Oral Pathol* 1967;23:806-10.
175. Carlsson J, Sundqvist G. Evaluation of methods of transportation and cultivation of bacterial specimens from infected root canals. *Oral Surg Oral Med Oral Pathol* 1980;49:451-4.
176. Pantera EA, Zambon JJ, Shih-Levine M. Indirect immunofluorescence for the detection of *Bacteroides* species in human dental pulp. *J Endod* 1988;14:218-23.
177. Möller ÅJR. Microbiological examination of root canals and periapical tissues of human teeth: methodological studies. Akademiforlaget, Goteborg, 1966.
178. Ng Y, Spratt D, Sriskantharajah S, Gulabivala K. Evaluation of protocols for field decontamination before bacterial sampling of root canals for contemporary microbiology techniques. *J Endod* 2003;29:317-20.
179. Reit C, Molander A, Dahlén G. The diagnostic accuracy of microbiologic root canal sampling and the influence of antimicrobial dressings. *Endod Dent Traumatol* 1999;15:278-83.
180. Molander A, Reit C, Dahlén G. Microbiological evaluation of clindamycin as a root canal dressing in teeth with apical periodontitis. *Int Endod J* 1990;23:113-8.
181. Strachan T, Read AP. PCR-based DNA cloning and DNA analyses. In: Strachan T, Read AP. *Human Molecular Genetics*. New York: Bios Scientific Publishers; 1996. p. 129-45.
182. Zambon JJ, Haraszthy VI. The laboratory diagnosis of periodontal infections. *Periodontol* 2000 1995;7:69-82.
183. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;11:266-73.

184. Molander A, Lundquist P, Papapanou PN, Dahlén G, Reit C. A protocol for polymerase chain reaction detection of *Enterococcus faecalis* and *Enterococcus faecium* from the root canal. *Int Endod J* 2002;35:1-6.
185. Siqueira JF, Rôças IN. PCR methodology as a valuable tool for identification of endodontic pathogens. *J Dent* 2003;31:333-9.
186. Siqueira JF, Rôças IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2004;97:85-94.
187. Rôças IN, Siqueira JF, Santos KRN. Association of *Enterococcus faecalis* with different forms of periradicular diseases. *J Endod* 2004;30:315-20.
188. Rôças IN, Jung I, Lee C, Siqueira JF. Polymerase chain reaction identification of microorganisms in previously root-filled teeth in a South Korean population. *J Endod* 2004;30:504-8.
189. Baumgartner JC, Siqueira JF, Xia T, Rôças IN. Geographical differences in bacteria detected in endodontic infections using polymerase chain reaction. *J Endod* 2004;30:141-4.
190. Dakin HD. On the use of certain antiseptic substances in the treatment of infectious wounds. *Brit Med J* 1915;2:318-20.
191. Walker A. A definite and dependable therapy for pulpless teeth. *J Am Dent Assoc* 1936;23:1418-25.
192. Grossman LI, Meiman BW. Solution of pulp tissue by chemical agents. *J Am Dent Assoc* 1941;28:223-5.
193. Lewis P. Sodium hypochlorite in root canal therapy. *J Fla Dent Soc* 1954;24:30.
194. Raphael D, Wong TA, Moodnik R, Borden BG. The effect of temperature on the bactericidal efficiency of sodium hypochlorite. *J Endod* 1981;7:330-4.
195. Pashley EL, Birdsong NL, Bowman K, Pashley DH. Cytotoxic effects of NaOCl on vital tissues. *J Endod* 1985;11:525-8.
196. Estrela C, Estrela CR, Barbin EL, Spano JC, Marchesan MA, Pecora JD. Mechanism of action of sodium hypochlorite. *Braz Dent J* 2002;13:113-7.
197. Shih M, Marshall FJ, Rosen S. The bactericidal efficiency of sodium hypochlorite as an endodontic irrigant. *Oral Surg* 1970;29:613-9.

198. Spångberg L, Engström B, Langeland K. Biologic effects of dental materials. 3. Toxicity and antimicrobial effect of endodontic antiseptics in vitro. *Oral Surg* 1973;36:856-71.
199. Rutberg, M, Spångberg E, Spångberg L. Evaluation of enhanced vascular permeability of endodontic medicaments in vivo. *J Endod* 1971;3:347-51.
200. Ellerbruch ES, Murphy RA. Antimicrobial activity of root canal medicament vapors. *J Endod* 1977;3:189-93.
201. Cunningham WT, Joseph SW. Effect of temperature on the bactericidal action of sodium hypochlorite endodontic irrigant. *Oral Surg* 1980;50:569-71.
202. Harrison JW, Hand RE. The effect of dilution and organic matter on the antibacterial property of 5.25% sodium hypochlorite. *J Endod* 1981;7:128-32.
203. Smith JJ, Wayman BE. An evaluation of the antimicrobial effectiveness of citric acid as a root canal irrigant. *J Endod* 1986;12:54-8.
204. Nikolaus BE, Wayman BE, Encinas E. The bactericidal effect of citric acid and sodium hypochlorite on anaerobic bacteria. *J Endod* 1988;14:31-4.
205. Foley DB, Weine FS, Hagen JC, deObarrio J. Effectiveness of selected irrigants in the elimination of *Bacteroides melaninogenicus* from the root canal system: an in vitro study. *J Endod* 1983;9:236-41.
206. Harrison JW, Wagner GW, Henry CA. Comparison of the antimicrobial effectiveness of regular and fresh scent Clorox. *J Endod* 1990;16:328-30.
207. Ohara P, Torabinejad M, Kettering JD. Antibacterial effects of various endodontic irrigants on selected anaerobic bacteria. *Endod Dent Traumatol* 1993;9:95-100.
208. Jeanson MJ, White RR. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. *J Endod* 1994;20:276-8.
209. Kuruvilla JR, Kamath MP. Antimicrobial activity of 2.5% sodium hypochlorite and 0.2% chlorhexidine gluconate separately and combined, as endodontic irrigants. *J Endod* 1998;24:472-6.
210. Sen BH, Safavi KE, Spångberg LS. Antifungal effects of sodium hypochlorite and chlorhexidine in root canals. *J Endod* 1999;25:235-8.

211. Cambuzzi JV, Marshall FJ. Molar endodontic surgery. *J Canad Dent Assoc* 1983;1:61-6.
212. Skillen WG. Morphology of root canals. *J Am Dent Assoc* 1932;19:719-35.
213. Coffae KP, Brilliant JD. The effect of serial preparation versus nonserial preparation on tissue removal in the root canals of extracted mandibular human molars. *J Endod* 1975;1:211-4.
214. Klayman SM, Brilliant JD. A comparison of the efficacy of serial preparation versus Giromatic preparation. *J Endod* 1975;1:334-7.
215. Walton RE. Histologic evaluation of different methods of enlarging the pulp canal space. *J Endod* 1976;2:304-11.
216. Richman MJ. The use of ultrasonics in root canal therapy and root resection. *J Dent Med* 1957;12:12-8.
217. Cunningham WT, Martin H, Pelleu G, Stoops D. A comparison of antimicrobial effectiveness of Endosonic and hand root canal therapy. *Oral Surg* 1982;54:238-41.
218. Martin H, Cunningham WT. Endosonic endodontics: the ultrasonic synergistic system. *Int Dent J* 1984;34:198-203.
219. Martin H, Cunningham WT. Endosonics-the ultrasonic synergistic system of endodontics. *Endod Dent Traumatol* 1985;1:201-6.
220. Walmsley AD, Williams AR. Effects of constraint on the oscillatory pattern of Endosonic files. *J Endod* 1989;15:189-94.
221. Lumley PJ, Walmsley AD. Effect of precurving on the performance of Endosonic K files. *J Endod* 1992;18:232-6.
222. Ahmad M, Roy RA, Kamarudin AG. Observations of acoustic streaming fields around an oscillating ultrasonic file. *Endod Dent Traumatol* 1992;8:189-94.
223. Ahmad M, Roy RA, Kamarudin AG, Safar M. The vibratory pattern of ultrasonic files driven piezoelectrically. *Int Endod J* 1993;26:120-4.
224. Ahmad M, Roy RA, Kamarudin AG. Variations in power output of the Piezon[®]-Master 400 ultrasonic endodontic unit. *Int Endod J* 1994;27:26-31.
225. Ahmad M, Roy RA. Some observations on the breakage of ultrasonic files driven piezoelectrically. *Endod Dent Traumatol*. 1994;10:71-6.

226. Roy RA, Ahmad M, Crum LA. Physical mechanisms governing the hydrodynamic response of an oscillating ultrasonic file. *Int Endod J* 1994;27:197-207.
227. <http://www.ultrasonics.com/uemagnet.htm>
228. Ingle JJ. Standardized endodontic technique utilizing newly designed instruments and filling materials. *Oral Surg* 1961;14:83-91.
229. <http://www.ultrasonics.com/uepiezo.htm>
230. Cameron JA. The use of ultrasound in the cleaning of root canals: a clinical report. *J Endod* 1982;8:472-4.
231. Chenail BL, Teplitsky PE. Endosonics in curved root canals. *J Endod* 1985;11:369-74.
232. Chenail BL, Teplitsky PE. Endosonics in curved root canals. Part II. *J Endod* 1988;14:214-7.
233. Kielt LW, Montgomery S. The effect of Endosonic instrumentation in simulated curved root canals. *J Endod* 1987;13:215-9.
234. Lim LC, McCabe JG, Johnson MR. SEM evaluation of sonic and ultrasonic devices for root canal preparation. *Quint Int* 1987;18:793-7.
235. Miserendino LJ, Miserendino CA, Moser JB, Heuer MA, Osetek, EM. Cutting efficiency of endodontic instruments. Part III. Comparison of sonic and ultrasonic instrument systems. *J Endod* 1988;14:24-30.
236. Calhoun G, Montgomery, S. The effects of four instrumentation techniques on root canal shape. *J Endod* 1988;14:273-7.
237. Yamaguchi M, Matsumori M, Ishikawa H, Sakurai T, Nakamura H, Naitoh M, Shiojima M, Kikuchi A. The use of ultrasonic instrumentation in the cleansing and enlargement of the root canal. *Oral Surg* 1988;65:349-53.
238. Haikel Y, Allemann C. Effectiveness of four methods for preparing root canals: A scanning electron microscope evaluation. *J Endod* 1988;14:340-5.
239. Bolanos OR, Sinai IH, Gonsky MR, Srinvasin R. A comparison of engine and air-driven instrumentation methods with hand instrumentation. *J Endod* 1988;14:392-6.

240. Baker MC, Ashrafi SH, Van Cura JE, Remeikis NA. Ultrasonic compared with hand instrumentation: a scanning electron microscope study. *J Endod* 1988;14:435-40.
241. Pugh RI, Goerig AC, Glaser CG, Luciano WJ. A comparison of four endodontic vibratory systems. *J Acad Gen Dent* 1989;37:296-301.
242. Biffi JCB, Rodrigues HH. Ultrasound in endodontics: a quantitative and histological assessment using human teeth. *Endod Dent Traumatol* 1989;5:55-62.
243. Walker TL, delRio CE. Histological evaluation of ultrasonic and sonic instrumentation of curved root canals. *J Endod* 1989;15:49-59.
244. Yahya AS, ElDeeb ME. Effect of sonic versus ultrasonic instrumentation on canal preparation. *J Endod* 1989;15:249-53.
245. Loushine RJ, Weller RN, Hartwell GR. Stereomicroscopic evaluation of canal shape following hand, sonic, and ultrasonic instrumentation. *J Endod* 1989;15:417-21.
246. Tang MPF, Stock CJR. The effects of hand, sonic, and ultrasonic instrumentation on the shape of curved root canals. *Int Endod J* 1989;22:55-63.
247. Ahmad M, Pitt Ford TR. A comparison using macroradiography of canal shapes in teeth instrumented ultrasonically and by hand. *J Endod* 1989;15:339-44.
248. Ahmad M, Pitt Ford TR. Comparison of two ultrasonic units in shaping simulated curved canals. *J Endod* 1989;15:457-62.
249. McCann JT, Keller DL, LaBounty GL. Remaining dentin/cementum thickness after hand or ultrasonic instrumentation. *J Endod* 1990;16:109-13.
250. Moorer WR, Wesselink PR. Factors promoting the tissue dissolving capability of sodium hypochlorite. *Int Endod J* 1982;15:187-96.
251. Griffiths BM, Stock CJR. The efficiency of irrigants in removing root canal debris when used with an ultrasonic preparation technique. *Int Endod J* 1986;19:277-84.
252. Cameron JA. The synergistic relationship between ultrasound and sodium hypochlorite: a scanning electron microscope evaluation. *J Endod* 1987;13:541-5.

253. Fischer G. Local Anesthesia. Berlin: Herman Meuser, 1919.
254. Jorgenson NB, Hayden J. Sedation, Local, and General Anesthesia in Dentistry. 2nd ed., Philadelphia: Lea and Febiger, 1967:62-73.
255. Malamed SF. Local anesthesia, 2nd ed. St. Louis: CV Mosby Co., 1986:195-9.
256. Gutierrez JH, Garcia J. Microscopic and macroscopic investigation on results of mechanical preparation of root canals. Oral Surg 1968;25:108-16.
257. Davis SR, Brayton SM, Goldman M. The morphology of the prepared root canal: a study utilizing injectable silicone. Oral Surg 1972;34:642-8.
258. Leys EJ, Griffen AL, Strong SJ, Fuerst PA. Detection and strain identification of *Actinobacillus actinomycetemcomitans* by nested PCR. J Clin Microbiol 1994;32:1288-94.
259. Schneider SW. A comparison of canal preparations in straight and curved root canals. Oral Surg 1971;32:271-5.
260. Goodman A, Reader A, Beck F, Melfi R, Meyers W. An in vitro comparison of the efficacy of the serialization technique versus a serialization/ultrasonic technique in human mandibular molars. Master's Thesis, The Ohio State University, 1983.
261. Lev R, Reader A, Beck F, Melfi R, Meyers W. An in vitro comparison of the efficacy of the step-back technique versus a step-back/ultrasonic technique in human mandibular molars. Master's Thesis, The Ohio State University, 1985.
262. Haidet J, Reader A, Beck F, Meyers W. An in vivo comparison of the efficacy of the step-back technique versus a step-back/ultrasound technique in human mandibular molars. Master's Thesis, The Ohio State University, 1987.
263. Archer R, Reader A, Nist R, Beck M, Meyers WJ. An in vivo evaluation of the efficacy of ultrasound after step-back preparation in mandibular molars. Master's Thesis, The Ohio State University, 1990.
264. McComb D, Smith DC. A preliminary scanning electron microscope study of root canals after endodontic procedures. J Endod 1975;1:238-42.
265. McComb D, Smith DC, Beagrie GS. The results of in vivo endodontic chemomechanical instrumentation-a scanning electron microscopic study. J Brit Endod Soc 1976;9:11-8.
266. Bolanos OR, Jensen JR. Scanning electron microscopy comparisons of the efficacy of various methods of root canal preparation. J Endod 1980;6:815-822.

267. Fairbourn DR, McWalter GM, Montgomery S. The effect of four preparation techniques on the amount of apically extruded debris. *J Endod* 1987;13:102-8.
268. Keudell K, Conte M, Fujimoto L, Ernest M, Berry HG. Microorganisms isolated from pulp chambers. *J Endod* 1976;2:146-8.
269. Matsumiya S, Kitamura M. Histo-pathological and histo-bacteriological studies of the relationship between the condition of sterilization of the interior of the root canal and the healing process of periapical tissues in experimentally infected root canal treatment. *Bull Tokyo Dent Coll* 1960;1:1-19.
270. Harrison JW, Baumgartner JC, Svec TA. Incidence of pain associated with clinical factors during and after root canal therapy. Part 1. Inter-appointment pain. *J Endod* 1983;9:384-7.
271. Yguel-Henry S, Vannesson H, von Stebut J. High precision, simulated cutting efficiency measurement of endodontic root canal instruments: influence of file configuration and lubrication. *J Endod* 1990;16:418-22.
272. Abou-Rass M, Piccinino MV. The effectiveness of four clinical irrigation methods on the removal of root canal debris. *Oral Surg* 1982;54:323-8.
273. Chow TW. Mechanical effectiveness of root canal irrigation. *J Endod* 1983;9:475-9.
274. Harrison JW. Irrigation of the root canal system. *Dent Clin N Am* 1984;28:797-808.
275. Trope M, Grossman LI. Root canal culturing survey: single-visit endodontics. *J Endod* 1985;11:511-13.
276. Bender IB, Seltzer S, Turkenkopf S. To culture or not to culture? *Oral Surg Oral Med Oral Pathol* 1964;18:527-40.
277. Torneck C. Therapeutic armamentarium. In: Clark JW, ed. *Clinical Dentistry*. Vol. 4, Chap. 4. New York: Harper and Row; 1976. p. 4-5.
278. Product insert: liquid dental transport medium (LDT). Anaerobe Systems. Morgan Hill, CA. 1992.
279. Goodman AD. Isolation of anaerobic bacteria from the root canal systems of necrotic teeth by the use of a transport solution. *Oral Surg Oral Med Oral Pathol* 1977;43:766-70.
280. Shabahang S, Goon WW, Gluskin AH. An in vivo evaluation of the Root ZX[®] electronic apex locator. *J Endod* 1996;22:616-8.

281. Pagavino G, Pace R, Baccetti T. A SEM study of the in vivo accuracy of the Root ZX[®] electronic apex locator. J Endod 1998;24:438-41.
282. Meares WA, Steiman HR. The influence of sodium hypochlorite irrigation on the accuracy of the Root ZX[®] electronic apex locator. J Endod 2002;28:595-8.
283. El Ayouti A, Weiger R, Lost C. The ability of the Root ZX[®] apex locator to reduce the frequency of overestimated radiographic working length. J Endod 2002;28:116-9.
284. Brunton PA, Abdeen D, MacFarlane TV. The effect of an apex locator on exposure to radiation during endodontic therapy. J Endod 2002;28:524-6.
285. Pratten DH, McDonald NJ. Comparison of radiographic and electronic working lengths. J Endod 1996;22:173-6.
286. <http://store.tulsadental.com/catalog/>
287. Iqbal MK, Maggiore F, Suh B, Edwards KR, Kang J, Kim S. Comparison of apical transportation in four Ni-Ti rotary instrumentation techniques. J Endod 2003;29:587-90.
288. Alaçam T, Demirtola N, Misirligil A, Ayhan N, Gokay O. In vivo comparison of antimicrobial effectiveness of conventional and ultrasound activated irrigation techniques in root canal therapy. Bull Tokyo Dent Coll 1987;1:19-22.
289. Baker NA, Eleazer PD, Averbach RE, Seltzer S. Scanning electron microscope study of the efficacy of various irrigating solutions. J Endod 1975;1:127-35.
290. Senia ES, Marshall FJ, Rosen S. The solvent action of sodium hypochlorite on pulp tissue of extracted teeth. Oral Surg 1971;31:96-103.
291. Krell KV, Johnson RJ, Madison S. Irrigation patterns during ultrasonic canal instrumentation. Part I. K-type files. J Endod 1988;14:65-8.
292. Jahde EM, Himel VT, Weir J. A comparison of short-term periapical responses to hand and ultrasonic file overextension during root canal instrumentation in the *Macaca fascicularis* monkey. J Endod 1987;13:388-91.
293. <http://www.kerrdental.com/GURU/Ask/FAQ/Endodontics/Ultrasonics.html>
294. Cameron JA. The use of ultrasound for the removal of the smear layer. The effect of sodium hypochlorite concentration; an SEM study. Austral Dent J 1988;33:193-200.

295. Cameron JA. The effect of ultrasonic endodontics on the temperature of the root canal wall. *J Endod* 1988;14:554-9.
296. Eriksson AR, Albrektsson T. Temperature threshold levels for heat-induced bone tissue injury: a vital-microscopic study in the rabbit. *J Prosthet Dent* 1983;50:101-7.
297. Akpata ES. Total viable count of microorganisms in the infected dental pulp. *J Dent Res* 1974;53:1330-3.
298. McGurkin-Smith R, Trope M, Caplan D, Sigurdsson A. Reduction of intracanal bacteria using GT[®] rotary instrumentation, 5.25% NaOCl, EDTA, and Ca(OH)₂. *J Endod* 2005;31:359-63.
299. Portenier I, Waltimo T, Ørstavik D, Haapasalo M. The susceptibility of starved, stationary phase, and growing cells of *Enterococcus faecalis* to endodontic medicaments. *J Endod* 2005;31:380-6.
300. Williams AR, Stafford DA, Hughes DE. Ultrasonic dispersal of activated sludge flocs. *J Appl Bacteriol* 1970;33:656-63.
301. Stumpf PK, Green DE, Smith FW. Ultrasonic disintegration as a method of extraction of bacterial enzymes. *J Bacteriol* 1946;59:487-91.
302. Williams AR. *Ultrasound: biological effects and potential hazards*. London: Academic Press; 1983.
303. Yumita N, Nishigaki R, Umemura K, Morse PD, Swartz HM, Cain CA, Umemura S. Sonochemical activation of hematoporphyrin: an ESR study. *Radiat Res* 1994;138:171-6.
304. Pitt WG, McBride MO, Lunceford JK, Roper RJ, Sagers RD. Ultrasonic enhancement of antibiotic action on Gram-negative bacteria. *Antimicrob Agents Chemother* 1994;38:2577-82.
305. Qian Z, Sagers RD, Pitt WG. Investigation of the mechanism of the bioacoustic effect. *J Biomed Mater Res* 1999;44:198-205.
306. Joyce E, Phull SS, Lorimer JP, Mason TJ. The development and evaluation of ultrasound for the treatment of bacterial suspensions. A study of frequency, power, and sonication time on cultured *Bacillus* species. *Ultrason Sonochem* 2003;10:231-4.
307. Duckhouse H, Mason TJ, Phull SS, Lorimer JP. The effect of sonication on microbial disinfection using hypochlorite. *Ultrason Sonochem* 2004;11:173-6.

308. Littman SH. Evaluation of root canal debridement by use of a radiopaque medium. *J Endod* 1977;3:135-8.
309. Sidow SJ, West LA, Liewehr FR, Loushine RJ. Root canal morphology of human maxillary and mandibular third molars. *J Endod* 2000;26:675-8.
310. Weine FS. The C-shaped mandibular second molar: incidence and other considerations. Members of the Arizona Endodontic Association. *J Endod* 1998;24:372-5.
311. Chunn CB, Zardiackas LD, Menke RA. In vivo root canal length determination using the forameter. *J Endod* 1981;7:515-20.
312. Ingle J, Zeldow B. An evaluation of mechanical instrumentation and negative culture in endodontic therapy. *J Am Dent Assoc* 1958;57:471-6.
313. Reit C, Dahlén G. Decision making analysis of endodontic treatment strategies in teeth with apical periodontitis. *Int Endod J* 1988;21:291-9.
314. Wilson M. Susceptibility of oral bacterial biofilms to antimicrobial agents. *J Med Microbiol* 1996;44:79-87.
315. Nair PNR, Henry S, Cano V, Vera J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after “one-visit” endodontic treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2005;99:231-52.
316. Costerton JW, Geesey GG, Cheng GK. How bacteria stick. *Sci Am* 1978;238:86-95.
317. Nair PNR, Sjögren U, Figdor D, Sundqvist G. Persistent periapical radiolucencies of root-filled human teeth, failed endodontic treatments, and periapical scars. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1999;87:617-27.
318. Cunningham CJ, Senia ES. A three-dimensional study of canal curvatures in the mesial roots of mandibular molars. *J Endod* 1992;18:294-300.
319. Walker RT. Root form and canal anatomy of mandibular second molars in a southern Chinese population. *J Endod* 1988;14:325-9.
320. Windley W, Teixeira F, Levin L, Sigurdsson A, Trope M. Disinfection of immature teeth with a triple antibiotic paste. *J Endod* 2005;31:439-43.

321. Lawrence JR, Wolfaardt GM, Korber DR. Monitoring diffusion in biofilm matrices using confocal laser microscopy. *Appl Environ Microbiol* 1994;60:1166-73.
322. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu Rev Microbiol* 1995;49:711-45.
323. Spratt DA, Pratten J, Wilson M, Gulabivala K. An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. *Int Endod J* 2001;34:300-7.
324. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999;284:1318-27.
325. Jung IL, Seo MA, Fouad AF, Spångberg LSW, Lee SJ, Kim HJ, Kum KY. Apical anatomy in mesial and mesiobuccal roots of permanent first molars. *J Endod* 2005;31:364-8.
326. Farzaneh M, Abitbol S, Lawrence HP, Friedman S. Treatment outcome in endodontics-the Toronto study. Phase II: initial treatment. *J Endod* 2004;30:302-9.
327. Noiri Y, Ehara A, Kawahara T, Takemura N, Ebisu S. Participation of bacterial biofilms in refractory and chronic periapical periodontitis. *J Endod* 2002;28:679-83.