NOVEL *EX VIVO* BIOFILM MODEL:

A COMPARATIVE STUDY OF ROOT CANAL DISINFECTION

by

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Abstract

Bacteria, organized as biofilms within the root canal, can cause apical periodontitis (AP). It has been shown that microorganisms harbouring in the anatomical irregularities of the root canal system, such as fins, grooves, or isthmuses after treatment, decrease the outcome prognosis of endodontic therapy. Therefore, adequate removal of these microorganisms is essential for the prevention and treatment of AP. While difficulties in disinfection have been recognized, limited data are available to directly assess the effectiveness of bacteria removal by treatment. Thus, the aim of this study is to develop a standardized *ex vivo* biofilm model, closely resembling the *in vivo* clinical situation, to quantify and compare the efficacy of hand, rotary nickel-titanium (NiTi) and self-adjusting file (SAF) instrumentation in the removal of biofilm bacteria.

Thirty-six extracted single-rooted human teeth with an ovoid cross-section canal were selected. Each tooth was split longitudinally and a 0.2 mm wide by 0.3 mm deep groove was placed in the apical 2 to 5 mm of the canal. After growing mixed bacteria biofilm inside the canal under anaerobic condition, the split halves were reassembled in a custom block, creating apical vapour lock. Teeth were randomly divided into 3 treatment groups (n = 10 per group) using: (1) hand stainless steel (SS) K-file; (2) ProFile NiTi rotary instrumentation; and (3) SAF. Irrigation consisted of 10 ml 3% sodium hypochlorite and 4 ml 17% ethylenediaminetetraacetic acid. Six teeth received no treatment. Areas of the canal inside and outside the groove were examined using a scanning electron microscope.

Within the groove, a smaller area remained occupied by bacteria after the use of SAF than ProFile and SS K-file (3.25%; 19.25%; 26.98%) ($P < .05$). For all groups, significantly

more bacteria were removed outside the groove than inside ($P < .05$). No statistical differences were found outside the groove ($P > .05$).

Although all techniques equally removed bacteria outside the groove, SAF significantly reduced more bacteria from within the apical groove. No technique was able to completely remove the bacteria. The biofilm model represents a potentially useful tool for future study of root canal disinfection.

Preface

All thesis work was completed by Dr. Jimmy S. Lin. The relative research contribution by Dr. Lin was 85%. The research abstract has been published online in the *Journal of Endodontics* 2012:38;e23, and presented at the American Association of Endodontists 2012 Annual Session in Boston.

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List of Abbreviations

AP ..... apical periodontitis

BHI ..... brain heart infusion

CHX ..... chlorhexidine (digluconate)

DNA ..... deoxyribonucleic acid

EDTA ..... ethylenediaminetetraacetic acid

EPS ..... extracellular polymeric substance(s)

NaOCl ..... sodium hypochlorite

NiTi ..... nickel titanium

PTD ..... post-treatment disease(s)

QS ..... quorum sensing

SAF ..... self-adjusting file

SEM ..... scanning electron microscopy

SRS ..... stratified random samplling

SS ..... stainless steel

WL ..... working length

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Dedication

To my parents, thank you for your unwavering love, support, and encouragement. I could not have asked for anything more. To UBC Faculty of Dentistry and Faculty of Graduate Studies, thank you for allowing me this wonderful opportunity to pursue the profession that I truly cherish. And to all my teachers and fellow residents, thank you for sharing your knowledge and experience, for you have taught me so much and made every step of this journey of learning so memorable.

Chapter 1: Introduction

Colonizing microorganisms such as those found in the infected root canal space are present either as free-floating (planktonic) single cells or attached to each other or to the root canal walls to form (sessile) biofilms. Whereas planktonic microorganisms can be more readily eliminated by a variety of different methods, the removal of sessile biofilm bacteria from the root canal remains a major challenge (Haapasalo *et al*. 2005; Ricucci and Siqueira 2010a). A biofilm is a community of microorganisms embedded in an extracellular polysaccharide matrix and attached to a solid surface. It has been accepted that within this community the biofilm bacteria express different phenotypes, often with different characteristics, than do the same bacteria in their planktonic state (Costerton *et al*. 1999). Notable among these differences is the increased resistance to antimicrobial agents that can be 100- to 1,000-fold greater for a species in a mature biofilm relative to that same species grown planktonically (Ceri *et al*. 1999). Microbial invasion of the root canal system can eventually lead to pulpal necrosis and apical periodontitis (AP). As the bacteria in the necrotic root canal grow mostly in sessile forms, the success of endodontic treatment will depend on the effective elimination of such biofilms.

Currently, the eradication of the microbial infection is accomplished mainly through mechanical instrumentation and chemical irrigation. While mechanical preparation of the infected root canal has been shown to be most effective in reducing the number of bacteria, it alone is unreliable in achieving adequate disinfection (Bystrom and Sundqvist 1981; Orstavik and Haapasalo 1990). Irrigation allows for cleaning beyond what might be achievable through instrumentation as it enhances further bacterial elimination, facilitates necrotic tissue removal, and prevents packing of infected debris apically (Haapasalo *et al*. 2005).

Nonetheless, the anatomic complexities of the root canal system present physical constraints that pose a serious challenge to adequate root canal disinfection using current available techniques such that residual bacteria are often found in areas such as fins, isthmuses, ramifications, deltas, accessory and lateral canals, and dentinal tubules (Peters *et al* 2001; Nair *et al*. 2005; Susin *et al*. 2010). Recently, a new instrumentation and irrigation device, the Self-adjusting file (SAF) system, was introduced by ReDent-Nova (Metzger *et al*. 2010a). Different from the traditional nickel-titanium (NiTi) rotary files, the SAF system uses a hollow reciprocating instrument which allows for simultaneous irrigation throughout the mechanical preparation. When inserted into the root canal, the manufacturer claims that the SAF is capable of adapting itself to the canal shape three dimensionally (Metzger *et al*. 2010b). The instrument is used in a transline (in-and-out) motion and the abrasive surface of the lattice threads promotes a uniform removal of dentin (Metzger *et al*. 2010a). Siqueira *et al*. (2010) found that preparation of long oval canals with the SAF was more effective in reducing intracanal *Enterococcus faecalis* counts.

The majority of endodontic biofilm studies have been conducted using models with monospecies bacterial cultures grown on membranes, glass or plastic, either under continuous or frequent supply of nutrients, ranging from a few hours to a few days old (Dunavant *et al*. 2006; Duggan and Sedgley 2007; Chai *et al*. 2007; Brandle *et al*. 2008; Williamson *et al*. 2009; Chavez de Paz *et al*. 2010). Most biofilm models employed thus far do not adequately reflect the complexity of the root canal anatomy, and neither do they simulate the clinical situation. Therefore, it is of importance to develop multispecies *ex vivo* biofilm models resembling *in vivo* endodontic biofilms for studying root canal disinfection. The aim of this study is (1) to introduce a novel *ex vivo* multispecies biofilm model in

extracted single-rooted teeth with a standardized groove in the apical root canal and (2) to use the model to test the efficacy of hand, rotary NiTi and SAF instrumentation in biofilm bacteria removal.

Chapter 2: Review of the Literature

2.1 Etiology of Endodontic Disease

Apical periodontitis (AP) is an inflammatory disease that affects the periradicular tissues surrounding the root. While AP may be caused by chemical and physical factors, various classic studies have firmly established the microbial etiology of the infected root canal system as being the primary cause of the disease (Kakehashi *et al.* 1965; Moller 1966; Bergenholtz 1974; Sundqvist 1976). Infections of endodontic origin arise as a result of the pulpal invasion by indigenous microflora residing within the oral cavity. Even though viruses (Glick *et al.* 1991; Li *et al.* 2009; Chen *et al.* 2009), fungi (Waltimo *et al.* 1997; Baumgartner *et al.* 2000; Waltimo *et al.* 2004; Gomes *et al.* 2007), archaea (Vianna *et al*. 2006; Vickerman *et al*. 2007), and protozoa (Baumgartner 2004) have been found as constituents of the normal oral microbiota and have been associated with irreversible pulpitis and AP, bacteria are by far the most dominant inhabitants of the oral cavity (Siqueira *et al*. 2008). It has been estimated that almost 700 bacterial species have been found in the oral cavity, with any one individual harboring 100-200 of these species (Paster *et al.* 2006). Approximately 10 billion bacterial cells reside in the oral cavity and, of those, over 60% still remain to be cultivated and characterized (Aas *et al.* 2005).

The microorganisms of the oral microbiota exist in a symbiotic relationship that otherwise do not cause harm to the dental pulp unless there is the breach in the host defense caused by advancing caries, coronal microleakage, trauma, iatrogenic restorative event, or periodontal disease (Rotstein & Simon 2000; Baumgartner 2004; Haapasalo *et al.* 2005; Siqueira *et al*. 2009a). When the innate and adaptive host immune systems of the pulp fail to accomplish sufficient clearance of the bacteria and their by-products, the advancement of

microbial colonization further into the pulp will lead to the progression of pulpitis, eventually causing pulp necrosis and the development of AP with associated periradicular bone destruction (Stashenko *et al.* 1998; Nair 2004; Hahn and Liewehr 2007a; Hahn and Liewehr 2007b; Hahn and Liewehr 2007c). As with other infectious diseases of the oral cavity, the interactions between the triad of host defense, microbial pathogenicity and their environment (i.e. nutrient availability, redox potential) are determining factors in the development and progression of AP (Stashenko *et al*. 1998).

2.1.1 Routes of Endodontic Infection

For bacteria to establish successfully as endodontic pathogens, they must overcome a series of barriers to infect the pulp. As mentioned previously, several routes of entry are possible but amongst them, dental caries represents the main pathway through which the bacteria enter the root canal system (Haapasalo *et al*. 2003). Although studies have established *Streptococcus mutans* as the chief pathogen associated with the onset of dental decay, its progression is invariably the result of a mixed microbial infection consisting of various acidogenic and aciduric bacteria including *Actinomyces, Bifidobacterium* and *Eubacterium* (Peterson *et al.* 2011). *Lactobacilllus* species have also been implicated in caries progression and are often found in the advancing front of the lesion (Edwardsson 1974). It has been shown that the bacteria invading the tubules under the caries can release antigenic by-products which reach the pulp even before frank pulpal exposure (Bergenholtz 1990). Thus, inflammatory reaction of the pulp starts well in advance of the bacterial invasion of the pulp tissue due to the initiation of the local immune response to the bacterial antigens (Pashley 1996).

At the earlier stages of dentin infection, pulpal inflammation is likely to be localized and reversible, as long as adequate dentinal thickness remains (Hoshino *et al*. 1992). This is because in vital teeth, the outward movements of dentinal tubular fluid and the contents occupying the tubules, such as odontoblast processes and the collagen fibrils they produce, can protect the pulp by reducing dentinal permeability to impede bacterial invasion (Michelich *et al*. 1978). Other phenomenon such as dentinal sclerosis, a hypermineralized layer formed in the translucent zone of the carious lesion due to the redeposition of calcifying salts, and the formation of tertiary dentin by activated odontoblasts, can also reduce dentin permeability (Pashley 1996). Host defense mechanisms, such as antibodies and components of the complement system, are present in the dentinal fluid of vital teeth to further assist against bacterial invasion of dentin (Okamura *et al.* 1979; Okamura *et al*. 1980). As the caries progress, the bacteria will eventually enter the heavily inflamed superficial layers of the pulp, but the core of the pulp remains relatively bacteria-free so long as its vitality remains (Haapasalo *et al*. 2003).

When the pulp becomes necrotic and the host defense is lost, bacterial invasion and colonization of the dentinal tubules and pulp proper becomes much more rapid (Nagaoka *et al.* 1995). The further egress of microorganisms and their toxic by-products from the necrotic root canal through apical, lateral, or furcation foramina, and communicating dentinal tubules can induce damages to the surrounding periodontal tissues, giving rise to AP (Haapasalo and Orstavik 1987; Safavi *et al.* 1990; Vertucci 2005). Bone destruction and the formation of apical lesion is a protective host defense response in limiting the spread of infection (Stashenko *et al.* 1992; Portenier *et al.* 2005). The localization of microbial challenge within the lesion allows for targeted recruitment of inflammatory cells to the

periradicular area to mount an immune response against the invading pathogens (Hou *et al*. 2000).

2.1.2 Microbiota of Endodontic Infection

A vital and healthy pulp resides in a sterile environment of the root canal system. When the pulp becomes infected and undergoes necrosis, it loses its blood supply and becomes a reservoir for the microorganisms and their by-products (Schein and Schilder 1975; Baumgartner 2004).

Endodontic disease is predominantly a polymicrobial infection, and studies have shown a correlation between apical lesion size to be proportional to the number of bacterial species in the root canal (Bystrom *et al.* 1987; Rocas and Siqueira 2008). Bacteria found in the infected canal fall into nine phyla, namely: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Spirochaetes*, *Synergistes*, TM7, and Sulphur River 1 (Munson *et al.* 2002; Siqueira *et* al. 2005; Saito *et al.* 2006; Sakamoto *et al.* 2006; Sakamato *et al*. 2007).

Although the oral environment harbours one of the highest indigenous microbata diversities in the human body, with over 500 microbial taxa identified (Sundqvist and Figdor 2005), only a relatively small number of those species are found in the infected canals (Sundqvist 1994). This indicates that the unique ecological conditions within the root canal system operate to select for the growth and survival of certain bacterial species. The main ecological factors that determine the composition of the microorganisms of the infected root canal are: oxygen tension (redox potential), nutrient availability, and competition or synergism between bacterial species (Siren *et al*. 2004).

Endodontic infections have traditionally been studied by means of culture approaches and, more recently, using molecular methods. Such studies have resulted in defining characteristic sets of species to be associated with the pathogenesis of specific forms (*primary* versus *persistent/secondary*) of AP.

2.1.2.1 Primary Endodontic Infection

Primary root canal infection is caused by microorganisms that initially invade and colonize the necrotic pulp tissue. It is characterized as an opportunistic polymicrobial infection consisting predominantly of Gram-negative anaerobic bacteria (Bergenholtz 1974; Sundqvist 1976; Fabricius *et al*. 1982). The origin of the initial participating microorganisms is likely from a subpopulation of bacteria involved in the early stages of pulp invasion, usually via advancing caries. With ensuing pulpal necrosis, the changes in the environment allow for the 'latecomers' to thrive and colonize the canal (Hahn and Liewher 2007a). Accordingly, depending on the microenvironment within different parts of the canal, variations in the distribution of differing microorganisms have been identified. Microorganisms found in the apical region are dominated by slow growing obligate anaerobes (Baumgartner and Falkler 199), whereas rapidly growing facultative anaerobes are predominantly found in the coronal aspect of the root canal (Fabricius *et al*. 1982). A recent study using molecular methods to explore the diversity of endodontic microbiota by cloning and sequencing 16S rRNA found a range of 3 to 21 (mean 10) species per infected canal (Ribeiro *et al*. 2011). Most frequently found bacteria are Gram-negative anaerobic rods such as *Prevotella*, *Porphyromonas*, *Dialister*, and *Fusobacterium* species (Haapasalo *et al*. 1986; Bae *et al*. 1997; Siqueira and Rocas 2002; Chavez de Paz and Villaneuva 2002; Gomes *et al*.

2005). Difficult to culture strict anaerobes such as the spirochetes have also been found in primary infections using molecular methods (Montagner *et al*. 2010).

Primary infections can manifest itself as chronic (asymptomatic) AP, or acute (symptomatic) AP (American Association of Endodontists 2009). Some chronic AP conditions can progress to chronic apical abscess which is characterized by the intermittent discharge of purulent exudate through a draining sinus tract, while certain acute AP can develop into an acute apical abscess with rapid onset of spontaneous pain, swelling, and the potential for the infection to spread to the head and neck spaces to establish a life threatening condition (Poeschl *et al.* 2011). Of the black-pigmented *Porphyromonas* species, so named for their ability to form brown and black colonies on blood agar plate, *P. gingivalis* and *P. endodontalis* have been found to be associated with acute (symptomatic) AP (Haapasalo *et al*. 1986).

Factors that determine the severity of endodontic infection may include: (1) virulence of bacterial species; (2) synergism and antagonism between bacteria; (3) population density; (4) environment-related expression of virulence factors; and (5) the ability of host defense to effectively respond to diverse microbial infection (Siqueira 2003). Even though Gram-negative anaerobic bacteria are the most common microorganisms in primary infections, several Gram-positive cocci and rods, as well as archaea, fungi and viruses have also been identified (Peciuliene *et al*. 2001; Vianna *et al*. 2009; Siqueira and Rocas 2009b; Li *et al*. 2009).

To summarize, the bacterial profile of primary AP is characterized as a heterogenic polymicrobial infection dominated by Gram-negative microorganisms. No single species can

be considered as the main pathogen but rather, multiple bacteria, in varying combinations, play the role in the causation and differing clinical manifestations of the disease.

2.1.2.2 Persistent and Secondary (Post-Treatment) Endodontic Infection

Following chemomechanical preparation of the infected root canal and the maintenance of asepsis with optimal obturation and coronal restoration, a favourable long-term treatment outcome can be expected. However, an unfavourable outcome can sometimes occur when there is the presence of persistent or secondary intraradicular infection (Siqueira 2001; Nair 2006). While refractory AP after initial treatment may be due to non-microbial factors such as true cysts, cholesterol crystals, foreign body reactions, or microbial causes of extraradicular origin, the major causative agent for unfavourable treatment outcome is due to intraradicular infections (Bhaskar 1966; Simon 1980; Brown and Theaker 1987; Nair and Schroeder 1984; Haapasalo *et al.* 1987; Nair *et al.* 1990; Sjogren *et al.* 1995; Nair *et al.* 1996; Nair *et al.* 1998; Lin *et al*. 2007).

By definition, persistent infections are caused by microorganisms remaining from members of primary infections that have survived in the canal after treatment. Secondary infections are caused by reinfection of microorganisms (i.e. via coronal leakage) that are not present in the canal during primary infections, but are introduced at some point in time after therapeutic intervention. At any particular point in time it may be difficult, if not impossible, to discern clinically and radiographically whether the apical lesion associated with a previously root canal treated tooth is caused by persistent or secondary infection. Therefore, the term post-treatment disease (PTD) may be more appropriate in describing both persistent and secondary infection.

As higher incidence of PTD is significantly associated with primary cases that have pre-operative radiolucency, it may be fair to infer that persistent infection, rather than secondary infection, as being the main intraradicular culprit for non-healing (Salehrabi and Rotstein 2004; de Chevigny *et al*. 2008a; Torabinejad *et al*. 2009). Likewise, the very high rate of success in the treatment of vital or non-infected cases lends further support to the assertion that persistent infections are the most common cause of PTD (Ng *et* al. 2007; Ng *et al*. 2008; de Chevigny *et al*. 2008b).

The last two decades have witnessed increasing emphasis on the role of coronal leakage and the belief that secondary infection is the most important cause of PTD (Khayat *et al.* 1993; Saunders and Saunders 1994; Ray and Trope 1995). However, Siqueira (2008) and others argued that should secondary infection due to coronal leakage be the most significant cause of PTD, the failure rates for the treatment of vital, necrotic, and retreatment cases should be similar and not be so different as observed (Chugal *et* al. 2007). Regardless of the differing sources of the causative microbial agents between persistent and secondary infection, the ecological pressure of both types of post-treatment endodontic infections necessarily dictates the selection of those microorganisms with the ability to manage and adapt to the harsh environment of the instrumented and filled canals (Shen *et al*. 2010a).

2.1.2.2.1 Role of *Enterococcus faecalis* in Post-treatment Disease

Whichever is the intraradicular source of the post-treatment endodontic disease, the microbiota exhibits a decreased diversity in comparison to primary infections. Failed root canals with apparently adequate treatment usually contain 1 to 5 species, while those that appeared inadequately treated can reach up to 30 species, a number more similar to primary infections (Pinheiro *et al*. 2003; Ribeiro *et al*. 2011). Regardless of using culture or

molecular methods, the predominant microorganisms identified are Gram-positive facultative anaerobes, with *Enterococcus faecalis* strains being the most common isolates (Siren *et al*. 1997; Molander *et al*. 1998; Sundqvist *et al*. 1998; Peciuliene *et al.* 2001; Portenier *et al*. 2003; Chavez de Paz 2004; Sedgley *et al*. 2004; Fouad *et al*. 20005; Williams *et al*. 2006; Sedgley *et al*. 2006; Zhou *et al*. 2010).

E. faecalis has a prevalence of up to 90% in PTD, and is 9 times more likely to be found than in primary infections (Molander *et al.* 1998; Sundqvist *et al*. 1998; Pinheiro *et al*. 2003; Rocas *et al*. 2004; Zoletti *et al*. 2006). Potential reasons that have been postulated for the ability of *E. faecalis* to survive in the ecologically challenging environment of the treated canal and to cause disease include: (1) ability to invade dentinal tubule and adhere to collagen (Haapasalo and Orstavik 1987; Orstavik and Haapasalo 1990; Love 2001); (2) production of gelatinase (Sedgley 2007); (3) metabolic adaptation under nutrient depravation (Portenier *et al*. 2005; Shen *et al*. 2010a; Shen *et al*. 2011); (4) resistance to irrigants and medicaments, and the presence of proton pumps (Bystrom *et al*. 1985; Haapasalo and Orstavik 1987; Safavi *et al*. 1990; Orstavik and Haapasalo 1990; Evans *et al*. 2002); (5) transfer of resistant gene pools via conjugation (Sedgley and Clewell 2004); (6) inhibition of medicaments by dentin (Haapasalo *et al*. 2000; Portenier *et al*. 2001; Portenier *et al*. 2002; Portenier *et al*. 2006; Haapasalo *et al*. 2007); (7) recruitment of polymorphonuclear neutrophils (Ma *et al*. 2011); and, not the least of which, (8) enhanced bacterial synergism through the formation of biofilm community (Tronstad and Sunde 2003; Svensater and Bergenholtz 2004; Siqueira and Rocas 2009c; Shen *et al*. 2009; Shen *et al*. 2010a; Shen *et al*. 2010b; Shen *et al*. 2011).

Although the precise role of *E. faecalis* in causing PTD has recently been questioned (Reynaud *et al*. 2005; Chavez de Paz 2007; Siqueira and Rocas 2009b), it at the very least remains a strong indicator species for its close association with the disease.

2.1.2.3 Biofilms in Endodontic Infection

Much in common with how most microorganisms are found in nature, the current trend in medicine recognizes that the majority of chronic endogenous infections are caused by mixed bacteria organized as members of metabolically integrated communities in the form of biofilms (Costerton 2007). Biofilms are dynamic sessile communities of interacting bacterial cells firmly attached to a surface and encased in a self-made matrix of extracellular polymeric substances (EPS) with water channels interconnecting the various communities (Costerton *et al*. 1994; Costerton *et al*. 1999; Socransky and Haffajee 2002; Donlan and Costerton 2002; Stoodley *et al*. 2002). The presence of water channel allows for the exchange of materials between bacterial cells and is believed to help coordinate the functions of the biofilm community (Costerton *et al.* 1994). Eighty-five percent by volume of the biofilm structure is made up of matrix material, while 15% is made up of cells. The glycocalyx matrix of the EPS surrounds the microcolonies and anchors the bacterial cells to the solid substrate (Fleming *et al*. 2007). Composition of the glycocalyx EPS consists of polysaccharides, proteins, nucleic acids and salts (Nivens *et al*. 2001; Whitchurch *et al*. 2002). There is a highly structured spatial distribution of bacterial microcolonies of different physiologic and metabolic states within the biofilm, and that these organized communities of bacteria can be endowed with properties that are greater than the sum of the individual units (Costerton *et al*. 1999).

Current estimates indicate that biofilm infections account for about 65% to 80% of the human infectious diseases (Costerton 2004). Similarly, it is increasingly apparent that oral bacteria causing caries, gingivitis and marginal periodontitis have the ability to grow and function as biofilms in the form of supragingival or subgingival dental plaque (Marsh 2005; Petersson *et al.* 2011). Parsek and Singh (2003) outlined the following criteria for defining infections caused by biofilms: (1) the bacteria are adherents of host surface structure; (2) examination of infected tissues shows bacterial microcolonies embedded in EPS; (3) the infection is generally confined locally, although dissemination of biofilms can occur secondarily; and (4) eradication of infection is difficult, if not impossible, with antimicrobial agents that otherwise would be effective in killing the bacteria in their planktonic state.

Given the difficulties in accomplishing biofilm eradication, the potential for bacteria in the root canal to organize themselves as such must be of great therapeutic interest in endodontics, as mounting evidence indicates that AP is a biofilm-induced disease (Svensater and Bergenholtz 2004; Chavez de Paz 2007; Siqueira and Rocas 2009c). Using light and scanning electron microscopes (SEM), Nair (1987) was possibly the first to identify clusters of "self-aggregating" or "co-aggregating" colonies of bacteria with a structure similar to dental plaque on the canal walls of infected roots. Other studies have demonstrated the ability of multiple bacteria to form a biofilm architecture on the root canal walls (Molven *et al.* 1991; George *et al*. 2005; Kishen *et al*. 2007; Chavez de Paz 2007; Distel 2007). In a recent clinical and histopathological study, Ricucci and Siqueira (2010b) found biofilm arrangements in the apical segment of 80% of canals with primary AP and 74% of canals with secondary or persistent AP, and that biofilm structures were more likely to be present in association with longstanding pathologic processes, such as large bony lesions and cysts.

Bacterial cells undergo a wide variety of morphological and physiological adaptations in response to chemical and physical changes in their environment. The unique characteristics of the biofilm consortia confer the bacteria enhanced capability to adapt to and survive in ecologically challenging environment. In endodontic infections, the main limiting factors within the root canal environment influencing bacterial colonization are oxygen and nutrient availability (Sundqvist 1992). After root canal treatment, further limiting factors imposed by the application of irrigants, medicaments and obturation materials necessarily alter the ecology for which the bacteria must adapt in order to survive (Chavez de Paz 2007). The polymicrobial community lifestyle affords several advantages to biofilm bacteria including: (1) protection from environmental threats such as host defenses, pH shifts, osmotic shock, desiccation, and antimicrobial agents; (2) creation of a broader habitat range for growth; (3) enhanced metabolic diversity and nutrient utilization via interaction through food webs; and (4) facilitation of genetic exchanges to optimize phenotypic and genotypic plasticity (Costerton *et al.* 1987; Costerton *et al*. 1995; Socransky and Haffajee 2000; Donland and Costerton 2002; Stoodley *et al*. 2002; Marsh 2003; Hall-Stoodley *et al*. 2004; Marsh 2005).

Communication between bacteria in biofilms is required in order for them to behave collectively as a consortium. Quorum sensing (QS) is a mechanism for which microorganisms can communicate with each other and is an integral component for regulating gene expression and modulating the phenotypic traits (Dunny and Leonard 1997; Withers *et al*. 2001). It involves the release of cell-to-cell signaling molecules called 'autoinducers' that increase in concentration as a function of cell density, which leads to the alteration of gene expression once a minimal stimulatory threshold concentration has been

reached (Miller and Bassler 2001). QS systems enable concerted behavioural decisions and have been shown to modulate bacterial virulence, alter metabolic states between growth and starvation phase, mediate bacterial resistance to antimicrobials, and direct biofilm architectural formation (Passador *et al*. 1993; Davis *et al*. 1998; Camilli and Bassler 2006).

To enhance virulence, QS enables the bacteria within the biofilm to mount a consolidated effort to overcome the host defense and further establish the infection (Kievit and Iglewski 2000). When cellular density increases as the biofilm communities grow, the increasing competition to available nutrient will lead to the starvation of bacteria. QS allows the crowded bacteria to communicate and slow down the metabolic activity to the stationary phase of growth to avert further increase in competition, permitting extended cell survival in the absence of nutrients (Lazazzera 2000). One of the earlier known examples of the role of cell-to-cell communication is the regulation of conjugation and plasmid transfer between *E. faecalis* bacteria (Dunny *et al*. 1978). The transfer of plasmid deoxyribonucleic acid (DNA) is one way that bacterial resistance to antibiotics can be established (Sedgley and Clewell 2004; Stewart and Costerton 2001). Ehrlic *et al*. (2005) further explored the diversity of biofilm bacteria interaction and coined the term 'bacterial plurality' as the reason for the inability of antibiotics to eradicate chronic infections. Bacterial plurality encompasses the concepts of supra-genome and the distributed-genome hypotheses which, in essence, give rise to the possibility of communal gene pool that enables the infecting bacterial population to display multiple phenotypes and genotypes (Erdos *et al*. 2003). This diversity provides the biofilm population, as a whole, the ability to persist in a hostile environment of immune host responses and antimicrobial interventions.

Recognizing that the majority of endodontic infections are likely biofilm-induced diseases much like other chronic infectious diseases of the human body, and that bacteria in the biofilm state are much more resistant to treatment than their planktonic counterparts, the focus of endodontic disinfection should, therefore, be to emphasize the eradication of the biofilm bacteria.

2.2 Management of Endodontic Infection

2.2.1 Goals of Endodontic Treatment

Since AP is an infectious disease caused by microorganisms colonizing, for the most part, as biofilms in the root canal system, the ultimate goal of endodontic treatment is to eliminate or, at least, sufficiently reduce the bacterial populations to levels that are compatible with periradicular tissue health (Orstavik 2003). For irreversibly inflamed pulp, endodontic treatment can be considered a prophylactic management since the apical region of the vital pulp is usually free from the invading bacteria, and the rationale for treatment is to prevent further infection of the pulp leading to the development of AP (Spangberg 2008).

Even though the goal of endodontic treatment is clear, the elimination of microorganisms from the infected canals is far from a simple task. Some studies have indicated that the prognosis of AP after endodontic treatment is poorer if bacteria are present at the time of root filling (Sjogren *et al.* 1997; Katebzadeh *et al*. 2000; Waltimo *et al*. 2005; Fabricius *et al*. 2006). Furthermore, other studies have shown that the presence of bacteria remaining in the canal after treatment plays a major role in the emergence of AP after treatment (Lin *et al*. 1992; Sundqvist *et al*. 1998; Molander *et al.* 1998; Pinheiro *et al*. 2003; Gomes *et al.* 2008). However, not all studies have been able to demonstrate significant

differences in healing between teeth with positive or negative cultures prior to obturation, it is nevertheless generally accepted that the prevention and healing of AP depends on the effective elimination of the microbes in the root canal system (Chugal *et al*. 2001).

2.2.2 Strategies in Endodontic Disinfection

2.2.2.1 General Strategies

The management of endodontic infections is based on concerted efforts by various host and treatment factors, such as: (1) host defense; (2) systemic antibiotic in specific situations; (3) chemomechanical debridement; (4) interappointment intracanal medicament; (5) root canal filling; and (6) coronal restorative seal (Haapasalo *et al*. 2003). Although all factors are important in the effective management of endodontic infection, mechanical preparation and chemical debridement of the root canal system is considered the major treatment modality for eliminating the causative microbial agents (Haapasalo *et al*. 2005).

2.2.2.2 Mechanical Disinfection: Instrumentation

Removal of vital and necrotic remnants of pulp tissues, microorganisms, and microbial toxins from the root canal system is essential for endodontic success (Gu *et al*. 2009). Mechanical instrumentation is often the first means of debridement during endodontic treatment of infected root canals. Although successful endodontic therapy depends on many factors, there is a general agreement that the physical effect of mechanical preparation of the root canal is one of the most critical stages in root canal disinfection (Hulsmann *et al*. 2005). It contributes to disinfection by disturbing or detaching the biofilms that adhere to the canal surfaces, and by removing the layer of infected dentin. Grossman (1970) stated that mechanical debridement as being the most important part of root canal

therapy. Schilder (1974) also considered cleaning and shaping as the foundation for successful endodontic treatment. Ideally, the root canal system should be free from microorganisms after biomechanical instrumentation and then sealed to prevent reinfection (Baugh and Wallace 2005). Instrumentation of the pulp-dentin complex essentially involves the removal of the diseased pulpal soft tissues and the infected dentinal hard tissues while preserving the structural integrity of the root dentin and the geometry of the canal anatomy (Hulsmann *et al*. 2005). Furthermore, instrumentation facilitates the delivery of irrigants, creates the space for the application of antimicrobial medicaments, and optimizes the canal dimensions for the placement of obturation materials (Schuping *et al*. 2000; Buchanan 2000). In essence, the quality of mechanical preparation is a predetermining factor on the efficacy of all subsequent procedures for endodontic treatment success (Peters 2004).

2.2.2.2.1 Impact of Instrumentation on Microbial Elimination

The effect of canal preparation on the reduction of bacteria has been researched extensively. Bystrom and Sundqvist (1981) studied how much manual instrumentation was able to achieve bacterial reduction using only physiological saline solution as irrigant. Their findings showed that although a significant bacterial reduction of 100- to 1000-fold was achievable after five sequential visits without the use of medicaments between each visit, obtaining a culture-free canal remained a challenge as 7 out of the 15 canals treated still contained cultivable bacteria. In a preceding study by Cvek *et al*. (1976), the antimicrobial efficiency of instrumentation using sterile saline and 0.5% or 2.5% sodium hypochlorite (NaOCl) in the treatment of necrotic permanent maxillary incisors had been compared. The authors found that the effect of instrumentation with sterile saline was limited for permanent teeth with mature root compared to the addition of even a low concentration of 0.5% NaOCl

during instrumentation. Interestingly, no statistical difference in antibacterial activity was found between irrigation using 0.5% versus 2.5% NaOCl solution. Their results showed that albeit mechanical instrumentation was helpful in the debridement of the canal, it alone was inadequate in the cleaning of the root canal.

With the advent of mechanized canal instrumentation, Dalton *et al*. (1998) compared the ability of 0.04 tapered nickel-titanium (NiTi) rotary preparation versus step-back stainless steel (SS) K-file hand instrumentation in reducing intracanal bacteria using sterile saline irrigation. All teeth with AP harboured cultivable bacteria at the beginning of treatment while the vital control teeth, diagnosed with irreversible pulpitis, were bacteria free. After successive canal enlargement using both techniques, a similar reduction in bacterial counts was observed between hand and rotary instrumentation, with only 28% of the teeth free of cultivable bacteria after the final preparation.

2.2.2.2.2 Size of Apical Enlargement and Microbial Elimination

The complex apical anatomy of the root canal presents a further challenge in root canal disinfection (Vertucci 2005). The apical portion of the root canal system has been shown to retain microorganisms that could potentially cause periodontal inflammation (Spangberg 2001). Owing to the various routes of communication via main and accessory foramina as well as the dentinal tubules, and the close proximity between the canal and the periodontal apparatus in the apical region, bacteria residing in the apical part of the root canal are in a privileged location to cause damage to the periradicular tissues (Siqueira 2001). The difficulty in disinfecting the hard to reach apical portion of the root canal and its potential impact on treatment outcome, therefore, warrants special attention in understanding how instrumentation can best eliminate microorganisms from this region.

During canal preparation, apical size enlargement has been shown to be critical in defining the successful of debridement. One guideline for determining the size of apical preparation advocated the enlargement to three file sizes larger than the initial file that bound in the canal (Weine 1972). However, Wu *et al.* (2002a) demonstrated that in canals with an oval cross section, 75% of the cases had the initial binding file contacting only one side of the apical canal wall and, even more disheartening, the initial file to bind failed to contact any portion of the apical wall in the remaining 25 % of the cases. In addition, other authors found enlarging the canal three sizes bigger than the initial file that binds were inadequate in removing the infected dentin circumferentially (Jou *et al.* 2004). Consequently, using the first file to bind for gauging the diameter of the apical canal and as a guide for determining final apical enlargement is unreliable. To help better predict the apical geometry clinically, recent studies have reported that initial coronal flaring before determining the apical size gave a more accurate assessment of the apical dimensions (Contreras *et al*. 2001; Tan and Messer 2002; Pecora *et al*. 2005).

Numerous studies have mostly confirmed that larger apical preparations demonstrated greater microbial reduction in the apical third of the canals. Using hand instrumentation, Orstavik *et al*. (1991) found that instrumentation to a #45 file facilitated the placement of calcium hydroxide intracanal medicament, and resulted in a decrease of bacterial growth by 10 fold. Their results corroborated those of another study which reported that, in conjunction with using calcium hydroxide, preparing the canal to a #40 file size decreased intracanal bacteria significantly more than smaller files (Sjogren *et al*. 1991). Dalton *et al*. (1998) showed that increasing file size preparation using rotary NiTi instruments resulted in greater bacterial reduction. Similarly, Usman *et al*. (2004) found that the size of final rotary NiTi

instrumentation was a good predictor of canal cleanliness. In their regression analysis, they demonstrated that the debris remaining in the apical third could mainly be predicted by the size of the rotary instrument; with enlargement to a size #40 file being much cleaner than a size #20 file.

2.2.2.2.3 Size of Apical Enlargement and Removal of Infected Dentin

In addition to debris and bacteria removal in the canal proper, it is also important for mechanical instrumentation to remove the infected circumferential dentin. It has been reported that bacteria may heavily invade the dentinal tubules to a depth of 200 μm or more (Haapasalo and Orstavik 1987; Love and Jenkinson 2002). Many *in vivo* studies found varying degrees of bacterial penetration in the dentinal tubules of infected canal (Armitage *et al*. 1983; Ando and Hoshino 1990; Sen *et al*. 1995; Love 1996); and one particular study showed that 70% of tubules had bacteria in them, some penetrating as far as the dentino-cemental junction (Matsuo *et al*. 2003). Nagaoka *et al*. (1991) observed that vital teeth were more resistant to tubular invasion but as time progressed, both vital and non-vital teeth showed greater depth of bacterial penetration. Furthermore, as the number of bacteria in the canal increased with time, their depth of invasion also increased (Akpata and Blechman 1982).

Uniform circumferential enlargement of the root canal by 200 μm has not been achieved with any contemporary technique; this appears to be an unattainable goal for current methods of mechanical instrumentation (El Ayouti *et al*. 2008; Paque *et al*. 2009). Peters *et al*. (1995) concluded that some bacteria superficially located in the tubules do not survive instrumentation and those that remain deeper in the tubules may be subsequently inactivated or of an insufficient number to cause pathology. However, in a later study, Peters

et al. (2001a) concluded that bacteria still present in the deeper levels of the tubules were of sufficient numbers that they could possibly cause recurrent infections.

2.2.2.2.4 Size of Apical Enlargement and Delivery of Irrigant

In addition to physically remove the infected soft and hard tissue contents, another goal of mechanical instrumentation is to enlarge the canal space sufficiently to allow for the delivery of irrigants to the apical regions of the canal. Research has shown that canals need to be enlarged to at least a #35 file size in order for the irrigants to adequately reach the apical one third (Salzgeber and Brilliant 1977). Other studies have demonstrated that enlarging the canal to a #40 file size maximizes the irrigant contact with debris and that preparation performed with smaller files did not allow for their adequate removal (Ram 1977; Chow 1983). In a randomized control clinical study using rotary NiTi instrumentation and 1.25% NaOCl irrigation to treat patients with radiographic and clinical signs of chronic AP, greater bacterial-free samplings were obtained from canals instrumented to a larger tip diameter (Shuping *et al*. 2000). The authors showed that NaOCl irrigation required a certain size canal before becoming beneficial in bacterial reduction. Based on their findings, they concluded that if the canal was not instrumented to an appropriate size, the whole purpose of using an antibacterial irrigant may be negated.

Mechanical instrumentation is important in physically removing the hard and soft infected tissue contents from the canal. Its role is made more difficult in the hard to reach apical area that is also most critical for treatment success. Research suggests that a larger apical preparation is needed to achieve better intracanal bacterial reduction, provide adequate removal of infected dentin with tubules invaded by the bacteria, and facilitate the delivery of irrigants.

2.2.2.3 Chemical Disinfection: Irrigation

Mechanical preparation using hand or rotary NiTi instrumentation can only be expected to act on the main body of the canal, leaving anatomically complex areas such as fins, isthmuses, and cul-de-sacs untouched after the completion of instrumentation (Wu and Wesselink 2001; Tan and Messer 2002; Wu *et al*. 2003; Peters 2004). Recently, microcomputed tomography has emerged as a powerful tool for three-dimensional evaluation of canal anatomy (Rhodes *et al*. 1999; Peters *et al*. 2000; Bergmans *et al.* 2001a). Using this technology to study the effects of instrumentation on canal anatomy, it was found that even in a relatively large and straight palatal canal of maxillary molar, about 50% of the canal surface remained without measurable change after NiTi rotary preparation (Peters *et al*. 2003). These uninstrumented areas may harbour tissue debris and microbes (Skidmore and Bjorndal 1971; Vertucci 1984; Ricucci and Siqueira 2010b), which will impede intimate adaptation of the filling materials (Wu *et al*. 2002b). Because of the limitations of mechanical instrumentation, the use of irrigation in root canal debridement is needed to allow for cleaning beyond what is achievable by root canal preparation alone (Gu *et al*. 2009).

2.2.2.3.1 Ideal Characteristics of Root Canal Irrigant

Haapasalo *et al*. (2010) outlined the desired properties of irrigating solutions. They include: (1) washing action to help debris removal; (2) reducing instrument friction; (3) breaking down organic tissue and dissolving inorganic matter; (4) being non-irritating to periradicular tissue; and (5) not adversely weakening the root structure. However, there is no one unique irrigant currently available that can meet all these requirements. Thus, contemporary irrigation protocols use two or more irrigants, with the sequential use of NaOCl followed by ethylenediaminetetraacetic acid (EDTA) being the most widely

employed regimen (Grande *et al*. 2006; Kishen *et al*. 2008). Recently, various combination products have been developed to improve the activity and function of the desired properties of irrigation solution (Torabinejad *et al*. 2003; Dunavant *et al*. 2006; Clarkson *et al*. 2006; Shen *et al*. 2009; Haapasalo *et al*. 2010; Stojicic *et al*. 2012). Improvements in the properties of root canal irrigants and their methods of delivery are all important areas of active research in endodontics (Zehnder 2006; Grande *et al.* 2006; Al-Hadlaq *et al*. 2006; Gu *et al.*2009).

2.2.2.3.2 Sodium Hypochlorite

The antiseptic property of hypochlorite has been recognized for close to a century. In World War I, a buffered 0.5% NaOCl solution had been used to cleanse infected wounds (Dakin 1915). The recognition that NaOCl has a wide-spectrum, non-specific, killing efficiency on microbes prompted its use as the principal endodontic irrigant as early as 1920 (Crane 1920; Walker 1936).

NaOCl, in concentrations from 0.5% to 6%, is currently the most widely used irrigation solution (Vianna *et al.* 2004; Stojicic *et al*. 2012). It encompasses many desirable properties of a main root canal irrigant and has therefore been described as the most ideal of all available disinfecting agents (Senia *et* al. 1975; Moorer and Wesselink 1982; Bystrom and Sundqvist 1983; Spangberg and Pascon 1998; Jeansonne and White 1994; Barnard *et al.* 1996; Zehnder 2006; Mohammadi 2008). Hypochlorite has the unique capacity to dissolve necrotic tissue (Grossman and Meiman 1941; Naenni *et al*. 2004) and the organic components of the smear layer (Koskinen *et al* 1980; Baumgartner and Mader 1987; Gutierrez *et al*. 1990; Haikel *et al*. 1994). It is a potent antimicrobial agent and kills bacteria in the dentinal tubules as well as sessile endodontic pathogens organized in biofilms (Orstavik and Haapasalo 1990; Spratt *et al*. 2001; Haapasalo *et al.* 2005). Inactivation of

endotoxin by hypochlorite has been reported (Sarbinoff *et al*. 1983; Silva *et al*. 2004); the effect, however, is less efficacious compared to that of calcium hydroxide root canal dressing (Tanomaru *et al*. 2003).

In water, NaOCl ionizes into Na^+ and hypochlorite ion, OCl^-, to establish an equilibrium with hypochlorous acid, HClO (Haapasalo *et al*. 2010). The active moiety of NaOCl is the hypochlorous acid which has been found to disrupt oxidative phosphorylation of cellular membrane as well as affecting the activity of DNA synthesis (Mckenna and Davie 1988; Barrette *et al*. 1989; Baumgartner and Cuenin 1992).

Recently, the effects of NaOCl on root canal biofilms have been studied more extensively. Comparing the cleaning efficacy of 1% or 6% NaOCl versus 2% chlorhexidine (CHX) against *in vitro E. faecalis* biofilms, the results showed that both concentrations of NaOCl proved statistically significant superior biofilm kill than CHX (Dunavant *et al*. 2006). An *in vitro* study on the effect of exposure to various irrigant solutions on apical dentin polymicrobial biofilms demonstrated a difference in the efficacy against bacteria by 3% and 6% NaOCl; the higher concentration being more efficacious (Clegg *et al*. 2006). The susceptibility of biofilm to NaOCl is also dependent on its age, metabolic state, and the composition of bacterial species. Studying the growth and susceptibility to different concentrations of NaOCl on mono- and dual- species *in vitro* biofilm of *F. nucleatum* or *P. micros* grown in polystyrene wells at 24 or 96 hr, Ozok *et al*. (2007) found that as the age of biofilms increased, so did their resistance to NaOCl. Furthermore, mixed species biofilms showed a greater time-dependent increase in synergy and resistance to NaOCl than single species biofilms.

Despite good *in vitro* results, the *in vivo* antimicrobial effectiveness of NaOCl is less promising. The decreased *in vivo* performance compared to the *in vitro* environment may be due to difficulties of NaOCl in penetrating the most apical and peripheral parts of the root canal system which often contains complex anatomical irregularities such as fins, anastomoses, and canal ramifications (Haapasalo *et al.* 2010). Also, the presence of inactivating substances such as periradicular exudates, pulp tissue remnants, microbial biomass, and the buffering capacity of dentin can counteract the effectiveness of NaOCl (Haapasalo *et al*. 2000).

Several clinical factors may help to increase the effectiveness of NaOCl irrigation, including: (1) application of mechanical and manual agitation (Martin 1976; Huang *et al*. 2008; McGill *et al*. 2008; Bhuva *et al*. 2010); (2) lowering the pH (Cotter *et al*. 1985); (3) increasing the temperature or concentration (Hand *et al.* 1978; Cunningham and Joseph 1980; Harrison and Hand 1981; Sirtes *et al.* 2005; Stojicic *et al*. 2010; Zhou *et al*. 2010); and (4) delivering sufficient volume and allowing for adequate contact time (Haapasalo *et al*. 2005). The ability to deliver the irrigant to the apical area is a function of the apical preparation dimension, needle size and depth of penetration, and needle tip design (Salzgeber and Brilliant 1977; Vinothkumar *et al*. 2007; Boutsioukis *et al*. 2009; Shen *et al*. 2010c; Boutsioukis *et al*. 2010). The use of a higher concentration of NaOCl and deeper needle tip insertion to improve irrigant delivery must be weighed against the potential risks of increased cytotoxicity and extrusion (Yesilsoy *et al.* 1995; Spencer *et al*. 2007; Hulsmann *et al*. 2009). Recently, negative pressure delivery devices have shown to help improve the volume of irrigant refreshment in the apical area and reduce the risk of extrusion (Nielsen and Baumgartner 2007; Desai and Himel 2009; Mitchelle *et al*. 2010).

While NaOCl does not possess all of the characteristics of an ideal endodontic irrigant, nevertheless it is the only solution capable of dissolving organic tissues and highly effective in direct killing of biofilm bacteria and, as such, is currently regarded as the main disinfecting solution used in conjunction with mechanical debridement of the infected canals.

2.2.2.3.3 Ethylenediaminetetraacetic Acid

Effective cleansing of the root canal system requires the removal of both the organic and inorganic substrates on the dentinal walls. One weakness of NaOCl is its inability to remove the inorganic component of the smear layer created during instrumentation (McComb and Smith 1975). The smear layer, consisting of dentin shavings, cellular debris, and pulpal remnants, can be characterized as having two separate zones: (1) a loose, superficial deposit, and (2) an attached stratum that extends into the dentinal tubules, forming occluding plugs (Sen *et al.* 1995). A SEM investigation revealed that instrumentation produced 1 to 2 μm thick smear layer on root canal walls (superficial deposit) which was pushed up to 40 μm into the tubules (occluding plugs) (Mader *et al*. 1984).

Considerable research efforts have been devoted to studying the effects of smear layer on endodontic treatment. The question of keeping or removing the smear layer had previously been controversial and much debated. Some researchers suggested that maintaining the smear layer may block the dentinal tubules to limit bacterial penetration and toxin penetration. By removing the smear layer and altering dentin permeability, they demonstrated deeper and more bacterial penetration into the tubules (Michelich *et al.* 1980; Pashley *et al.* 1981; Safavi *et al.* 1990). Pashley (1984, 1985) suggested that if the canals were inadequately disinfected, or if bacterial contaminations were to occur after canal preparation, the presence of smear layer might help prevent bacterial invasion of the dentinal

tubules. Furthermore, Drake *et al*. (1994) suggested leaving the smear layer intact in vital cases for the same reason of decreasing dentin permeability against bacterial invasion. However, Williams and Goldman (1985) demonstrated that the smear layer was not a complete barrier and could only delay, but not prevent, bacterial penetration. Those advocating for smear layer removal cite literature that shows: (1) it has a variable thickness and therefore acts as an unpredictable barrier (Cergeneux *et al*. 1987); (2) it contains bacteria, their by-products, and infected tissues debris, capable of causing inflammation (McComb and Smith 1975; Goldberg and Abramovich 1977; Cunningham and Martin 1982, Yamada *et al*. 1983); (3) it may act as a substrate for bacteria, thus facilitating deeper tubular penetration (George *et al*. 2005); (4) it may limit penetration and decrease the antimicrobial property of disinfecting agents (Outhwaite *et al*. 1976; Wayman *et al*. 1979; Bystrom and Sundqvist 1981, 1983, 1985; Haapasalo and Orstavik 1987); (5) it can serve as a barrier between obturation materials and canal walls, thereby compromising seal (Lester and Boyd 1977; White *et al*. 1984; Czonstkowsky *et al*. 1990; Yang and Bae 2002); and (6) its loose adherence to the canal walls can be a potential avenue for leakage, allowing for bacterial contaminant passage between the root filling and the dentinal walls (Mader *et al*. 1984; Cameron 1987; Meyron and Brook 1990). The current consensus is that the presence of smear layer after instrumentation serves more harm than good, and should therefore be eliminated prior to root canal obturation (Hulsmann *et al*. 2003).

Several chelating agents have been used in endodontics to remove the inorganic component of the smear layer. EDTA, a decalcifying chelator commonly available as a 17% neutralized solution, is the most frequently used irrigant to dissolve inorganic materials (Haapasalo *et al*. 2010). EDTA is a calcium complexing agent and removes the inorganic

dentinal debris, including hydroxyapatite, remaining on the root canal walls during preparation (Loel 1975; Baumgartner and Mader 1987). It has little or no effect on organic tissues and, despite some conflicting claims (Ohara *et al.* 1993), it alone does not exert antibacterial activity (Haapasalo *et al.* 2010). By removing the smear layer and opening the dentinal tubules, EDTA facilitates the penetration of disinfecting agents and enhance the antimicrobial efficiency of other irrigants, intracanal medicaments and sealers (Goldman *et al*. 1982; Haapasalo and Orstavik 1987; Orstavik and Haapasalo 1990; Torabinejad *et al*. 2002; Zehnder *et al*. 2005). However, it should be noted that the sequential use of NaOCl after EDTA has been shown to cause dentin erosion (Wei *et al*. 2011). The clinical sequelae of dentin erosion are unknown and warrant further investigation.

2.2.2.3.4 Other Irrigants

Chlorhexidine digluconate (CHX) is widely used as a disinfectant in dentistry because of its good antimicrobial action (Russell and Day 1993). It possesses a broad spectrum of antimicrobial activity (Oncag *et al*. 2003; Rosenthal *et al*. 2004), and has little toxicity (Jeansonne and White 1994; White *et al*. 1997; Kuruvilla and Kamath 1998). Various studies comparing the antibacterial effect of NaOCl versus 2% CHX against endodontic infection have shown little or no difference between their antimicrobial effectiveness (Vahdaty *et al.* 1993; Heling and Chandler 1998; Buck *et al.* 2001). However, CHX cannot replace NaOCl as it has no tissue-dissolving capability and is unable to remove or disrupt the biofilm structure (Baumgartner *et al*. 2007; Haapasalo *et al*. 2010).

Surface-active agents have been added to different types of irrigants to lower their surface tension and to improve their penetration in the root canal. Several studies reported that when surface-active agents were added to CHX, superior killing of biofilm bacteria was

accomplished by the combination products (Shen *et al.* 2009; Shen *et al.* 2011). QMiX, a new irrigation solution containing EDTA, CHX and a surface active detergent, with improved activity against *E. faecalis* and mixed plaque biofilms, has recently been introduced (Stojicic *et al*. 2012). Comparing QMiX with 1% and 2 % NaOCl as well as 2% CHX, the results showed that QMiX was superior to all solutions in killing plaque biofilm bacteria after 3 min of use (Stojicic *et al*. 2012).

Cleaning and shaping are important, interdependent steps, in root canal treatment. Canal preparation aims to mechanically remove the microbial biomass, diseased pulp tissues, and the infected dentin, while at the same time enlarging the canal to facilitate the delivery of irrigants. The goal of irrigation is to cleanse the canal by killing microorganisms and removing the organic and inorganic debris beyond what is achievable through instrumentation. While the knowledge concerning the etiology of endodontic infections, structure of microbial organization, physical complexity of canal anatomy, and biochemical environment of the root canal system has vastly expanded, predictable and complete removal of the infectious agents to treat root canal disease remains a challenge. Ongoing basic and translational research is needed to improve and optimize the use of existing techniques and products, and to develop innovative methods or materials, to attain the treatment goal of complete disinfection of the root canal system in teeth with AP.

2.2.2.4 Self-Adjusting File: A New System for Mechanical and Chemical Disinfection

The objectives of cleaning and shaping the root canal have been made easier to achieve with the advent of NiTi rotary file systems. Compared to the traditional files made of stainless steel (SS), the superelastic property of NiTi alloy enables these files to better preserve the location of the root canal axis at a greater preparation diameter and taper

(Esposito and Cunningham 1995; Bergmans *et al*. 2001b; Hulsmann *et al*. 2005), especially where curvatures are present (Esposito and Cunningham 1995; Pettiette *et al*. 1999; Kfir *et al*. 2004).

However, while the two-dimensional radiographic results of root canal fillings achieved after rotary NiTi instrumentation appear impressive, the third dimension of the canal geometry in the bucco-lingual axis is commonly forgotten (Spangberg 2001). In a relatively narrow canal with a round cross-section, cleaning and shaping of the canal may be more predictably achieved after sequential enlargements by SS hand files and NiTi rotary files. However, in flat oval-shaped canals, such as those found in the distal root of lower molars or upper and lower premolars, this goal is not easily achieved (Wu and Wesselink 2001; Wu *et al*. 2003). A comprehensive study by Wu *et al*. (2000) has shown that oval or flat canal morphology, as defined by the bucco-lingual dimension being greater than twice its mesio-distal width, is present in up to 25% of root canals, and may exceed 50% in certain root types. The greater bucco-lingual dimension is often not appreciated in the straight-on projection clinical radiographs (Vertucci 2005). The tendency for many hand and rotary instrumentation techniques is to produce round preparations (Vessey 1996; Hulsmann *et al*. 2011) with areas within the oval canal wall uninstrumented (Walton 1976; Gambill *et al*. 1996; Evans *et al*. 2001; Wu and Wesselink 2001; Ardilla *et al*. 2003, Bergmans *et al*. 2003), inevitably leaving infected debris and dentin in these regions.

Furthermore, canals with asymmetrical tear-shaped cross-sections, common in most roots with two canals joined by isthmus, or canals with irregular anatomy such as fins and cul-de-sacs, pose another challenge in cleaning and shaping because bacteria present in the form of biofilms have been identified in these anatomical irregularities (Nair *et al*. 2005).

The reported incidence of isthmuses in the mesial root of mandibular molars ranges from 54 to 89%, which are typically found in the middle to apical thirds of the canal (Hsu and Kim 1997). Not only are these areas difficult to access with instrument, instrumentation can actually further complicate cleaning by packing debris into these isthmus areas, hindering the access of irrigants into these regions (Paque *et al*. 2009). Endal *et al*. (2011) found that even with generous irrigation using solutions capable of dissolving organic and inorganic matter during and after rotary instrumentation, debris and bacteria packed into the isthmus areas cannot be prevented or removed.

Recently, an innovative file and irrigation system called the self-adjusting file (SAF; ReDent-Nova, Ra'anana, Israel) has been introduced with the potential of improving both instrumentation and irrigation, and to side-step some of the limitations of SS hand and rotary NiTi instrumentation in canals with irregular cross-sectional geometry (Metzger *et al*. 2010a). The SAF has a unique hollow file design conferring compressibility and flexibility and allowing for continuous irrigation. The cylindrical file with a pointed tip is composed of interconnecting NiTi lattices (Figure 4.6) and can be inserted into any canal previously prepared with a #20 K-file (Hof *et al*. 2010). When inserted into the canal, the instrument is claimed to conform itself to the canal shape, both longitudinally and cross-sectionally, providing a three-dimensional adaption (Hof *et al*. 2010). The SAF is operated using a transline (in and out) vibrating handpiece at 0.4 mm amplitude and 3,000 to 5,000 cycles per minute. The surface of the lattice threads is lightly abrasive, which allows for the removal of dentin with a back-and-forth grinding motion as the compressed file expands. An irrigation device with adjustable flow rate of delivery of up to 15 ml per min of solution is connected by a silicon tube to the hub of the file, enabling for a continuous flow of irrigant throughout

instrumentation (Figure 4.6). The manufacturer claims that a uniform removal of dentinal layer 60-75 μm thick circumferentially is achieved after 4 min of preparation, with the resulting apical size usually being at least equivalent to a #40 file (Metzger *et al*. 2010a; Metzger *et al*. 2010b).

Early studies have shown that the percent of root canal affected by the SAF method is larger than that achieved by current rotary instrumentation systems (Peters *et al*. 2010). It has also been reported that SAF is more capable in debris and smear layer removal (Metzger *et al*. 2010c). The durability of the fine delicate hollow file and its interconnecting NiTi lattices in terms of abrasivity and fracture resistance has also been demonstrated to be well beyond the intended clinical application (Hof *et al*. 2010). By adapting itself to the canal cross section and allowing for simultaneous irrigation, SAF has the potential of addressing the shortcomings of traditional hand and rotary file systems, especially in irregular and asymmetrical canals.

2.3 Studying Endodontic Infection: Using Biofilm Models

The task of disinfecting the root canal system is one of the most prominent challenges in endodontic therapy and as such has been the major focus in endodontics research. While the microbial etiology of endodontic infection has long been established (Kakehashi *et al.* 1965; Moller 1966; Bergenholtz 1974; Sundqvist 1976), only recently has there been the understanding that the vast majority of the colonizing microorganisms in the infected canal invariably grow and function as members of the metabolically integrated communities – or biofilms (Donlan and Costerton 2002; Costerton 2007).

Endodontic microbes, residing within the root canal anatomy as biofilms, are encased in a protective surface-adherent EPS such that they are much more difficult to remove and

more resistant to antimicrobial agents than the planktonic bacteria (Ricucci and Siqueira 2010a). In addition, several other factors enable bacterial biofilms to effectively evade treatment and survive. Bacteria located in ramifications, fins, isthmuses, and other anatomical irregularities are likely to escape the effects of instruments and irrigants used during chemomechanical procedures. Indeed, biofilms have been described as being present in the undebrided parts of the root canal system from surgically resected root apices (Nair *et al*. 2005). Furthermore, the progression of endodontic infection alters the nutritional and environmental status of the root canal system, resulting in a nutrient and oxygen depleted state to yield a challenging ecological niche for the surviving microbes (Sundqvist and Figdor 2003). The harsh condition is made even more severe for those bacteria that have escaped treatment procedures (Haapasalo *et al*. 2003). The biofilm mode of growth enables the bacteria to adapt and grow in unfavourable environment and nutrient deficient conditions (Grenier and Mayrand 1986). Accepting that endodontic infection is a biofilm disease and that biofilm bacteria are more resistant to treatment than their planktonic counterparts, it is essential for microbiological investigations to develop models to assess the effects of different disinfecting strategies in endodontics on biofilm bacteria eradication.

In vitro microbiological studies have focused on the efficiency of antimicrobial agents and disinfection strategies to remove biofilms grown in wells (Dunavant *et al*. 2006; Duggan and Sedgley 2007; George and Kishen 2007; Kishen *et al*. 2010; Shrestha *et al*. 2010); glass substrates (Williamson *et al*. 2009); membrane filters (Spratt *et al*. 2001; Hiraishi *et al*. 2010; Hope *et al.* 2010; Badr *et al*. 2011); polystyrene plates (Liu *et al*. 2010; Upadya and Kishen 2010); collagen-coated and non-coated hydroxyapatite discs (Shen *et al*.

2009; Shen *et al*. 2010a; Shen *et al*. 2010b; Pappen *et al*. 2010; Shen *et al*. 2011); and dentin samples (Distel *et al*. 2002; George *et al*. 2005; Kishen *et al*. 2006; Kowalski *et al*. 2006).

Few studies have used *ex vivo* models of growing biofilms in the canal of extracted human teeth. Due to the difficulties of growing biofilms in root canals, all of the studies thus far used monospecies *E. faecalis* biofilms as oppose to the multispecies biofilms found naturally in the oral cavity (Shabahang and Torabinejad 2003; George and Kishen 2008; Soares *et al*. 2010; Bhuva *et al*. 2010; Hope *et al*. 2010), without attempting to replicate commonly occurring anatomical irregularities such as fins and isthmuses where the biofilms can hide from the effects of disinfecting agents (Siqueira and Rocas 2008; Susin *et al*. 2010). Furthermore, an issue not addressed in most models until recently is the phenomenon of vapour lock occurring within the canal system which prevents unrestricted flow of irrigant to the apical regions that is crucial for thorough debridement (Moser and Heuer 1982; Chow 1983; Hsien *et al*. 2007; Tay *et al*. 2010). Vapour lock occurs as a result of gas entrapment occurring within a closed apical root system during irrigation which prevents optimal irrigant delivery and flow (Tay *et al*. 2010). With the exceptions of studies by Baumgartner and coworkers (Baumgartner and Mader 1987; Albrecht *et al*. 2004; Usman *et al*. 2004; Nielsen and Baumgartner 2007), few *ex vivo* studies have incorporated experimental set ups that truly produced a closed canal system designed to simulate the effect of gas entrapment (Fukumoto *et al*. 2006; Tay *et al* 2010). According to Gu *et al*. (2009) the conclusions derived from any root canal disinfection studies which did not properly take into account in the experimental design the effects of vapour lock must be interpreted with caution.

Microbiological sampling of the root canal is a commonly employed method to assess treatment effects in clinical studies (Bystrom and Sundqvist 1981, 1983; Dalton *et al*. 1998),

as well as in extracted teeth (Shabahang and Torabinejad 2003; George and Kishen 2008; Soares *et al*. 2010; Hope *et al*. 2010; Siqueira *et al*. 2010). However, a limitation of all sampling techniques is that only the planktonic or free-floating bacteria within the root canal system can be evaluated. Inaccessible regions of the root canal system such as, fins, isthmuses, and accessory canals cannot be adequately evaluated by microbiological sampling (Bhuva *et al*. 2010). Furthermore, quantification of biofilm by enumerating the Colony Forming Units may be problematic if the bacteria are sensitive to the culturing process, or are in a metabolic state non-conducive for growth in culture media. Recently, Shen *et al.* (2010b) demonstrated that in older, starved biofilms the bacteria were viable based on the confocal laser scanning microscopy patterns, but over 99% of those bacteria were not recoverable when removed from the viable biofilm and grown in a culture media.

The use of high-magnification electron microscopy has been employed for the morphological and structural characterization of microbial biofilms (Soares *et al*. 2010; Bhuva *et al*. 2010). The main disadvantage with this technique, other than the destruction of the specimen, is the need for extensive sample preparation steps such as fixation, dehydration, freeze- or critical point- drying, and vacuum sputtering. Although structural modifications in the biofilm architecture do occur during sample preparation, particularly in the overall collapse of matrix volume due to the dehydration process, the shape and dimension of the bacterial cells are maintained and be clearly studied by SEM (Sutton *et al*. 1994).

The potential for biofilm experimentation in endodontics has not been fully exploited. For example, the Zurich biofilm model (Guggenheim *et al*. 2004) is a well developed aerobic biofilm model derived from supragingival plaque. However, it is doubtful whether the

application of this model to the anaerobic ecological niches within the root canal space would be appropriate (Chavez de Paz 2007). Although the importance of developing standardized intracanal biofilm models for endodontic experiments has been well recognized (Gu *et al*. 2009), no study has yet published any models that sufficiently represent the *in vivo* conditions. Therefore, it is of importance in endodontic research to develop a versatile, standardized, biofilm root canal model closely resembling the *in vivo* environment that can be used for testing various modalities of root canal disinfection under clinically relevant conditions, and such that the effects of treatment on the biofilms can be quantitatively assessed.

Chapter 3: Aim and Hypothesis

3.1 Aim

Since bacteria organized as biofilms within the infected root canal cause AP, their removal is the primary objective of root canal treatment. Despite significant advancement in chemomechanical debridement techniques, eradication of all microorganisms from the root canal system remains a challenge. While clinical trials provide the highest level of evidence for studying the effectiveness of treatment, they are often not feasible due to ethical and logistical barriers. However, various platforms developed thus far for testing the efficacy of root canal disinfection do not adequately reflect the complex nature of the root canal anatomy or the biofilm bacteria residing within. Therefore, it is of importance to develop a standardized *ex vivo* biofilm model, closely resembling the complex *in vivo* conditions, for studying root canal disinfection.

The aim of the research is divided into two phases:

1. To develop a novel standardized *ex vivo* multispecies biofilm root canal model mimicking the clinical environment.
2. Employing the biofilm model to study the efficacy of root canal disinfection using a new file and irrigation system SAF, versus existing hand and rotary NiTi instrumentation.

3.2 Hypothesis

The SAF system, with its ability to adapt to the canal geometry and deliver continuous irrigation, is more capable than rotary NiTi and hand instrumentation in removing bacterial biofilm.

Chapter 4: Materials and Methods

4.1 PHASE 1: Development of Standardized *Ex Vivo* Biofilm Tooth Model

4.1.1 Standardization of Working Length and Tooth Selection

Thirty-six straight, single-rooted, human teeth of at least 19 mm in length with ovoid cross section were selected from a random pool of maxillary premolars and stored in 0.01% NaOCl solution until they were used. The canals were accessed and the length of the teeth was determined by inserting a size #10 SS K-file into the canal until file tip was just visible at the apical foramen. The reference cusp was reduced until each tooth measured 19 mm in length. Working length (WL) was defined as 1 mm short of the apical foramen at 18 mm.

4.1.2 Standardization of Apical Canal Dimension and Tooth Splitting

To facilitate the standardization of the apical canal geometry, the canal was hand instrumented to WL with a size #15 SS K-file followed by ProTaper S1 and S2 NiTi rotary files (Dentsply Tulsa Dental Products, Tulsa, OK). Dentin debris removal must be visible in at least the apical 4 mm portion of the S2 file in order for the tooth to be included. Under the dental operating microscope at 12 X magnification (Global Surgical, St. Louis, MO), grooves were made on the buccal and lingual surface of the tooth with a low-speed abrasive diamond disc (Brasseler, Savannah, GA) and a fine razor blade was used to split the tooth longitudinally through the centre of the canal in the bucco-lingual dimension. The split halves were examined under magnification to confirm that they can be reapproximated predictably (Figure 4.1).

Figure 4.1 Standardization of root length and apical canal geometry in split tooth. A human premolar tooth was made to 19 mm in length. After instrumentation with ProTaper S2 NiTi rotary file to facilitate the standardization of apical canal geometry, the tooth was split longitudinally in the bucco-lingual direction.

4.1.3 Standardization of Apical Groove

Under 20 X magnification (Global Surgical), a standardized groove with a dimension of 0.2 mm wide x 0.3 mm deep x 3.0 mm long, resembling a fin or isthmus, was placed in the canal wall of each half tooth 1 to 4 mm from the WL (2 to 5 mm from the apical foramen), using a modified microsurgical blade (G. Hartzell & Son, Concord, CA). The microsurgical blade has a width of 0.2 mm and a depth marking at 0.3 mm. Notches, 1 mm apart, were placed on the side of the root to reference the apical, middle and coronal thirds of the groove. The apical, middle and coronal thirds of the groove were defined as 1 to 2 mm, 2 to 3 mm and 3 to 4 mm short of the WL, respectively (Figure 4.2).

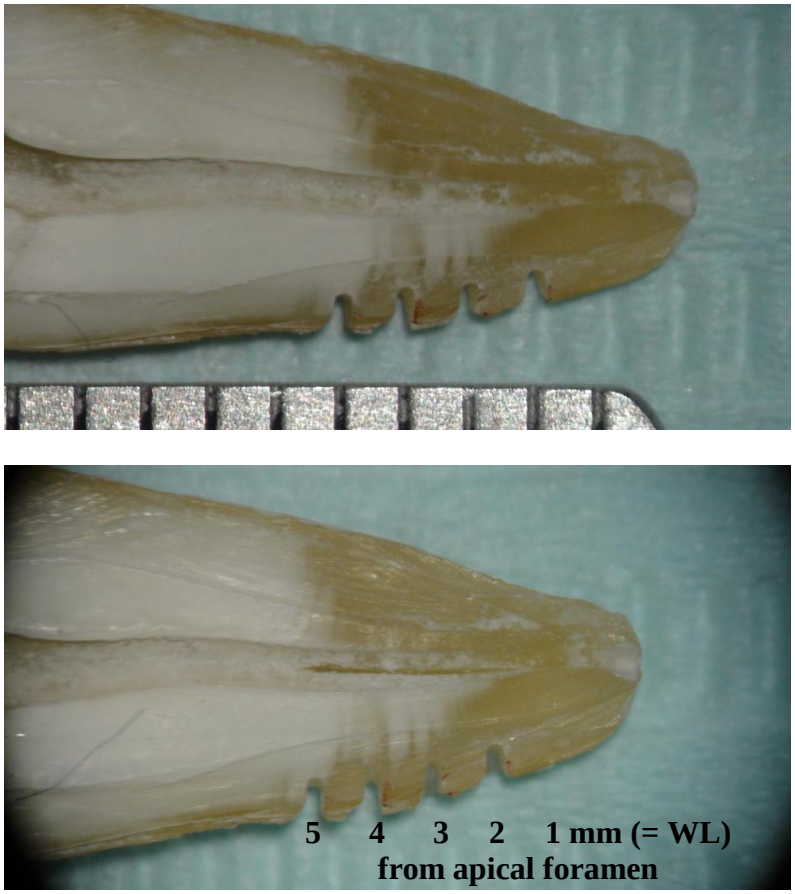

Figure 4.2 Standardization and placement of apical groove. Notches 1mm apart were made on the side of the split tooth at 2, 3, 4, and 5 mm from the apical foramen (above). An artificial groove, 0.2 mm wide X 0.3 mm deep X 3.0 mm long, resembling a fin or isthmus, was placed in the canal wall 2 to 5 mm from the apical foramen(or 1 to 4 mm from WL) (below). The apical, middle and coronal thirds of the groove were defined as 1 to 2, 2 to 3, and 3 to 4 mm short of the WL, respectively.

4.1.4 Standardization of Apical Concavity and Closed Apical System

The split halves of the tooth were reapproximated and 0.2 gm of utility wax (Coltene-Whaledent, Cuyahoga Falls, OH), formed into a round shape (approximately 5 mm diameter), was placed over the root tip to serve as a block out material. The tooth with the apical wax was encased in dental stone (Heraeus-Kulzer, Hanau, Germany) to form a custom block. To make the custom block, half of the split tooth and wax was covered in the first pour of the dental stone and, after setting, a thin layer of Vaseline (Unilever, Rotterdam, Netherlands) was used as a separator followed by a second pour covering the remaining half of the tooth and wax. After setting of the second pour, the dental stone was separated and the wax was removed to form a standardized apical concavity in the block. The custom block was used to help accurately reapproximate the split halves, secure the tooth during treatment, and simulate an *in vivo* closed apical system which provided resistance to irrigant flow by creating the apical vapour lock effect (Figure 4.3).

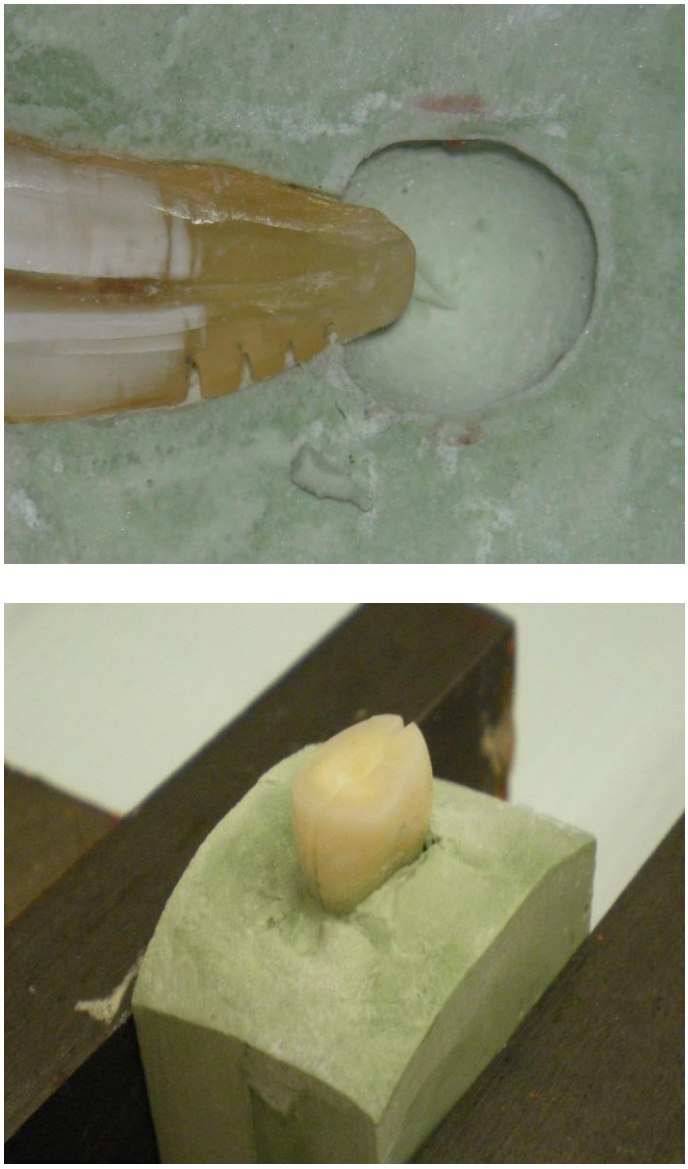

Figure 4.3 Standardization of apical concavity and closed apical system. The split halves of the tooth were reapproximated and secured with a custom block.

4.1.5 Growing Multispecies Biofilms in Root Canal

The split halves of the canal were each rinsed with 3 ml 17% EDTA for 3 min to remove the smear layer followed by a 10 mL wash using physiologic saline for 10 min. Each half canal was inoculated with human plaque bacteria in 3.5 mL Brain Heart Infusion (BHI; Difco, Detroit, MI) broth and stored in an anaerobic environment (AnaeroGen; Oxoid, UK) at 37°C for 4 weeks. Plaque samples were obtained from healthy donors who signed informed consent (Appendix A) approved by the University of British Columbia Clinical Research Ethics Board (CREB # H12-00907). The BHI growth medium was changed once weekly. After 4 weeks, a robust growth of multispecies biofilm, similar in appearance to those found *in vivo*, can be seen in the canal and confirmed at high magnifications with SEM (Figure 4.4 and Figure 4.5). Of note, hard-to-grow spirochetes were found in high numbers within the biofilm community (Figure 4.5). The significant features of the novel *ex vivo* split-tooth mixed human plaque bacteria biofilm model are listed in Table 4.1.

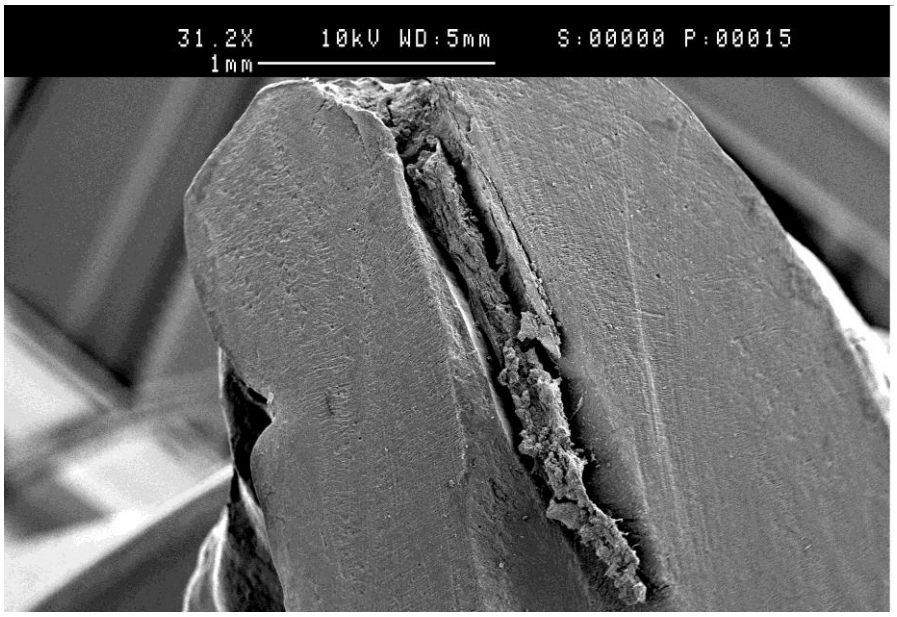

Figure 4.4 SEM image of bacterial biofilm model, 30 X magnification. Biofilm growth covering the canal after 4 weeks, using BHI under anaerobic environment.

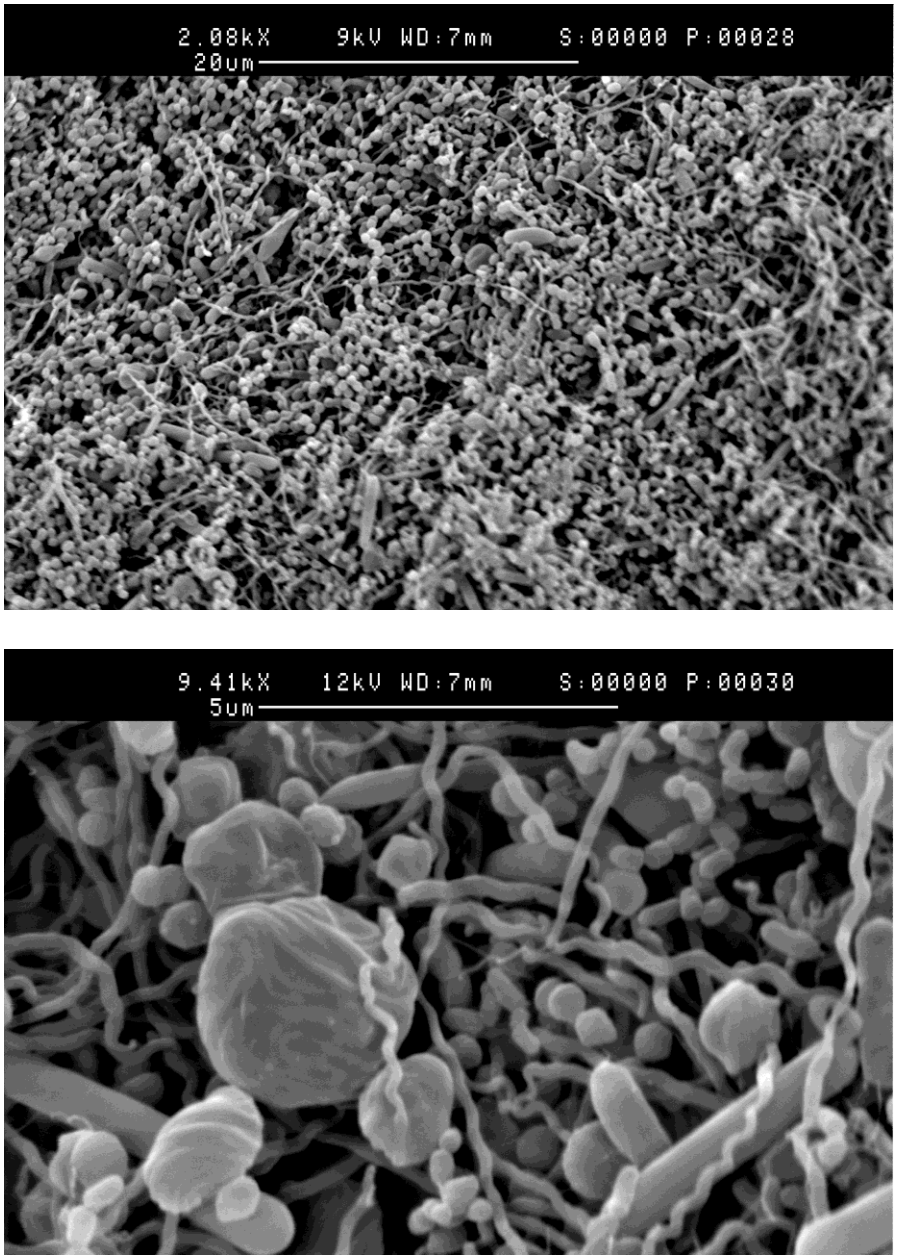

Figure 4.5 SEM image of bacterial biofilm model at 2000 X and 9000 X magnification. Robust multispecies bacteria, similar to those found *in vivo*, were observed in the biofilm. Of note, spirochetes were found in high numbers throughout the biofilm community.

Significant Features of *Ex Vivo* Biofilm Model

1. Standardization
    - Root length
    - Apical canal geometry
    - Apical groove (resembling anatomical fin or isthmus)
    - Apical concavity (resembling bone loss)
2. Human plaque multispecies anaerobic biofilm (resembling *in vivo* endodontics infection)
3. Closed apical system (vapour lock)
4. Simulate clinical treatment
5. Direction visualization and quantification of treatment effects
6. Versatility. Enable to study the efficacy of biofilm removal using various techniques

Table 4.1 Significant features of novel *ex vivo* split-tooth multispecies biofilm model.

4.2 PHASE 2: Studying Endodontic Disinfection, Using the Biofilm Tooth Model

The reassembled teeth in custom blocks were randomly divided into three treatment groups (n = 10 teeth per group) as followed: Group 1, step-back hand instrumentation using SS K-files; Group 2, crown-down rotary NiTi instrumentation with ProFile .04 taper files (Dentsply Tulsa Dental Products, Tulsa, OK); and Group 3, SAF system NiTi reciprocating files (ReDent-Nova, Ra'anana, Israel). Within each group, two extra teeth were used as controls and did not receive treatment. Treatments were performed by a clinician (JL) well versed in all three instrumentation methods, and in a manner simulating the clinical environment using rubber dam isolation.

4.2.1 Hand Instrumentation and Manual Dynamic Irrigation

SS K-files sequence #25, #30 and #35 were used with a reaming motion to instrument the canal to WL followed by shaping of the coronal half of the canal with Gates Glidden drills #2, #3, and #4. Further apical shaping was accomplished by employing the balanced-force technique with a #40 SS K-file as the master apical file to WL, then stepping back 1 mm shorter for each subsequent file sizes #45, #50 and #55 (i.e. 17, 16, and 15 mm respectively). A #15 file was used for recapitulation to WL in between each file. Using a syringe attached to a 30-gauge side-vented needle (Max-i-Probe; Dentsply Rinn, Elgin, IL), the canal was filled with 3% NaOCl solution during instrumentation and approximately 1 mL of the irrigant was exchanged after each recapitulation using the manual dynamic agitation technique. A further 3 mL of 3% NaOCl rinse, with the needle tip inserted without binding to within 3 mm of the apical foramen, was performed after the last instrument for a total of 10ml of NaOCl. A 2 min rinse with 4 mL of 17% EDTA was used as the final irrigant.

4.2.2 Rotary NiTi Instrumentation and Manual Dynamic Irrigation

Crown-down instrumentation technique was performed using ProFile .04 (Dentsply Tulsa Dental Products) NiTi rotary files in the sequence of #35, #30, reaching WL with #25, followed by apical enlargement at WL with #30, #35 and #40 as the master apical file. The same irrigation protocol as with hand instrumentation using syringe/needle manual dynamic agitation was employed: approximately 1 mL of 3% NaOCl was used between each instrument and 3 mL of 3% of NaOCl was delivered to within 3 mm of apical foramen after the last file for a total of 10 mL, followed by a 4 mL rinse with 17% EDTA for 2 min as the final irrigant.

4.2.3 SAF Instrumentation and Continuous Irrigation

The SAF was operated using KaVo GENTLEpower (KaVo, Biberach Riss, Germany) transline vibrating and reciprocating handpiece with RDT3 attachment head (ReDent-Nova) at a frequency of 4000 movements per minute and amplitude of 0.4 mm, according to the manufacturer's instruction. The file has a hollow design which allowed for continuous delivery of irrigants, supplied by a pump (Vatea; ReDent-Nova), throughout the instrumentation procedure (Figure 4.6). The SAF was inserted into the canal while vibrating and was gradually worked towards the WL with gentle pushing. The single file started as a narrow, compressed, shape which gradually expanded as the abrasive NiTi lattices removed dentin circumferentially during instrumentation (Metzger *et al*. 2010a). The file was operated with an in-and-out motion at the WL for 2 min with continuous irrigation using 3% NaOCl solution at a flow rate of 5 mL/min, for a total of 10 mL of NaOCl. A second cycle of 2 min of instrumentation was repeated with the SAF using continuous irrigation of 17% EDTA at a

flow rate of 2 mL/min (4 mL total). The resulting apical size after SAF instrumentation is usually at least equivalent to a size #40 file (Metzger *et al*. 2010b).

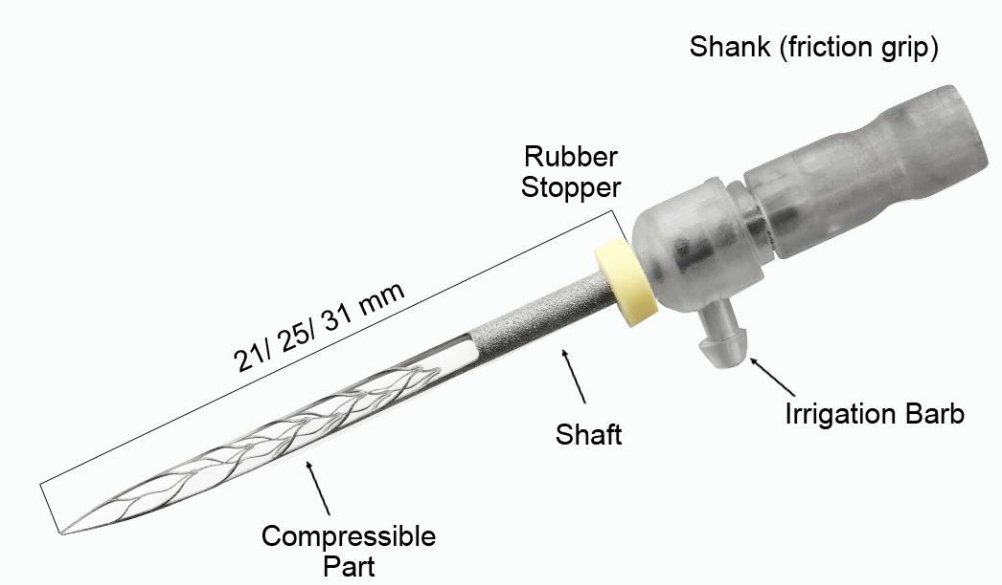

Figure 4.6 Self-adjusting file (SAF) instrumentation and irrigation system. The innovative hollow NiTi file is made of two longitudinal struts interconnected by abrasive compressible NiTi lattices. The file has an irrigation barb for attachment to the Vatea irrigation pump with adjustable flow rate. SAF is operated with a transline vibrating motion handpiece at 4000 cycles per min and, the hollow core enables the continuous delivery of irrigant throughout instrumentation. Photos courtesy of ReDent-Nova.

4.2.4 SEM Preparation and Stratified Random Sampling

After treatment, the teeth were disassembled and the split halves with the standardized apical groove were prepared for SEM evaluation. For each treatment group, SEM images were taken at the apical (1 to 2 mm from WL), middle (2 to 3 mm from WL) and coronal (3 to 4 mm from WL) thirds of the groove as well as their corresponding areas outside the groove. Stratified random sampling (SRS) technique was used as a mean to avoid bias in the acquisition of SEM images for evaluating biofilm removal. Randomization involved predetermining the sampling location within the groove at a low magnification (i.e. approximately 15 X), whereby the content details were not visible (Figure 4.7), then zooming into the middle of that area at higher magnifications for sampling (Figure 4.7, 4.8 and 4.9). Stratification entailed taking multiple images in a systematic manner in the area adjacent to the predetermined site (Figure 4.8). Within each third of the groove at the SRS predetermined location, a standardized image at 2000 X magnification was taken plus one adjacent image on each side (3 images per site) for evaluation (Figures 4.9). An additional 3 images at 2000 X magnification were taken outside the groove in the area adjacent to where the 3 images inside the groove were taken. For each group (10 teeth, or 20 split halves), 60 standardized 2000 X SEM images were acquired for each third of the groove, totaling 180 images for the entire groove. Similarly, 180 standardized images at 2000 X were taken in the corresponding areas outside the groove (Figures 4.10, 4.11). Another two teeth (or 4 split halves) for each group received no treatment and their SEM images were examined to ensure the presence of robust biofilm growth within the canal (Figure 4.5).

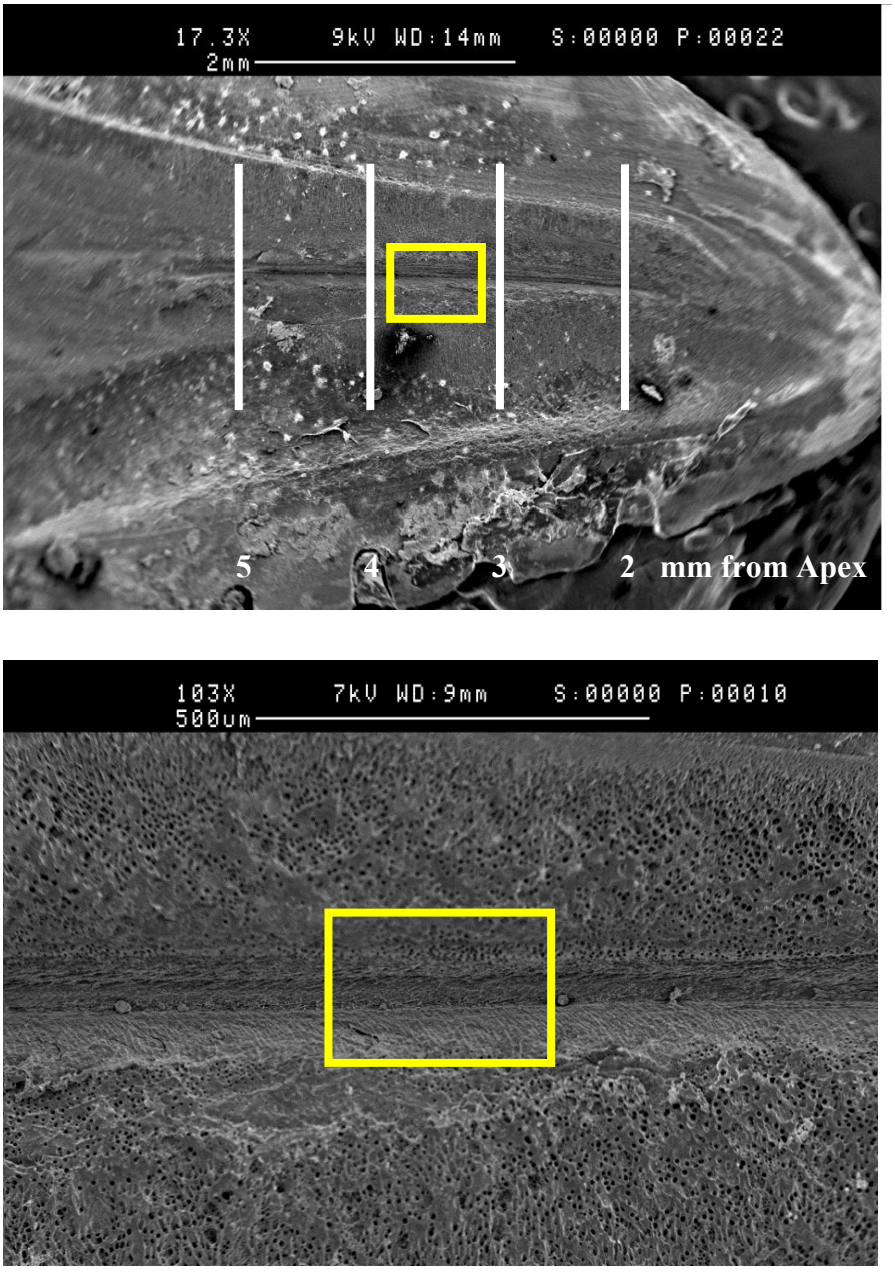

Figure 4.7 Stratified random sampling of SEM image. The sampling site of the groove is predetermined at low magnification (i.e. approximately 15 X) whereby the details of the groove content are not yet visible. Further magnification at 100 X, the groove content still not visible.

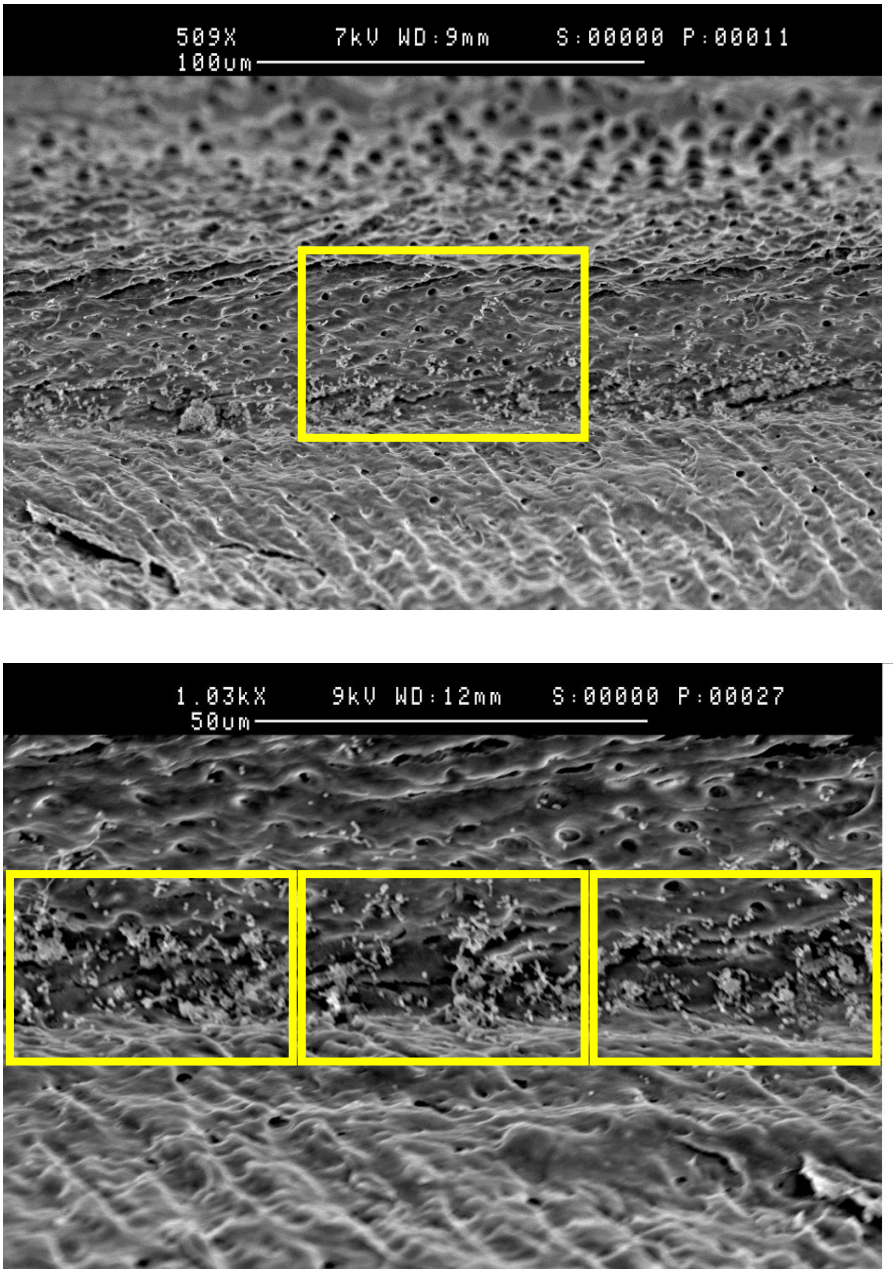

Figure 4.8 Stratified random sampling of SEM image. At 500 X magnification, the contents of the groove are becoming visible. At 1000 X magnification, the contents are clearly visible. Here, the areas from the middle of the image and on each adjacent side will be taken. This is stratification of the random sample site.

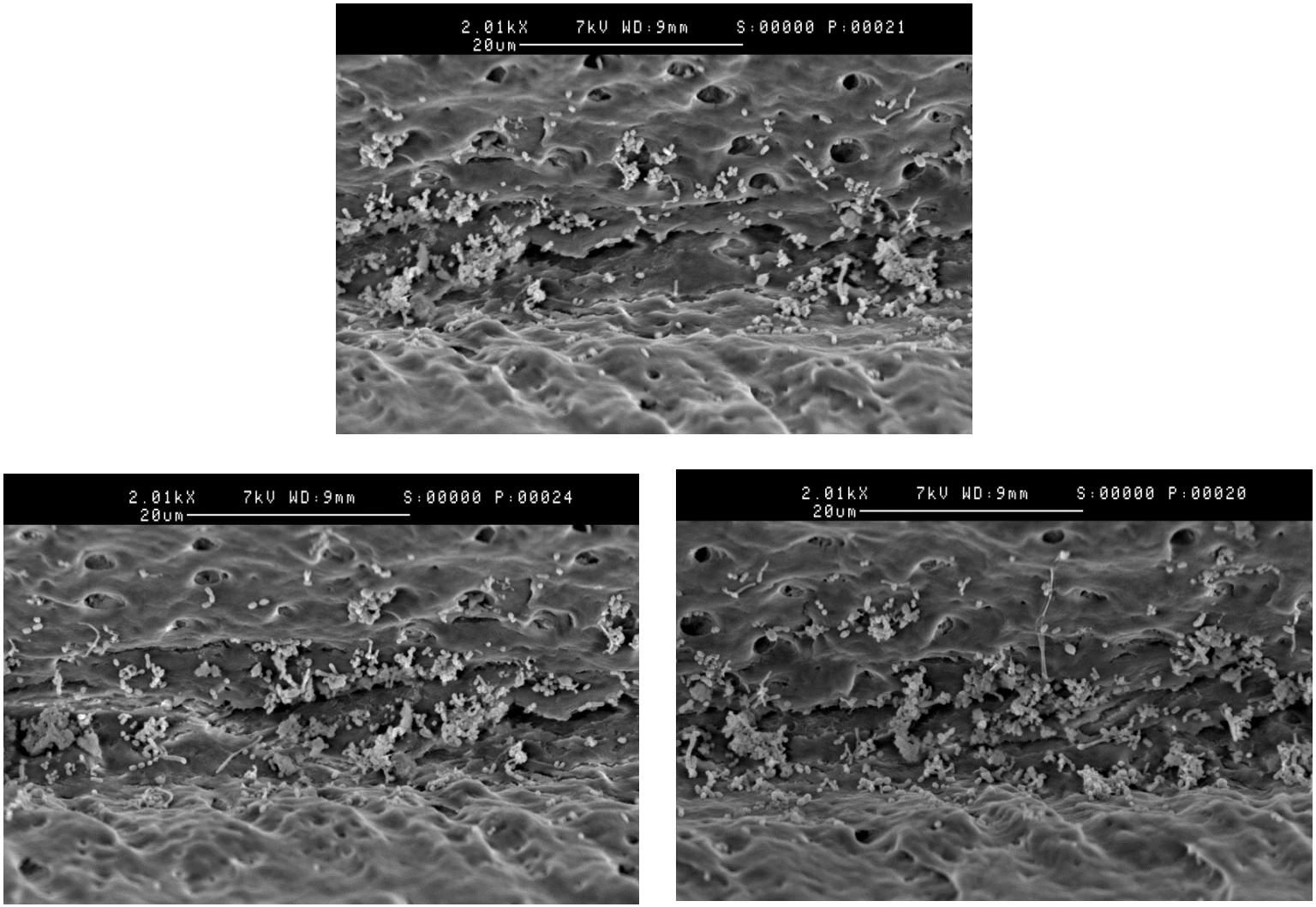

Figure 4.9 Standard SEM images at 2000 X obtained by stratified random sampling inside the groove for calculation of % bacteria remaining after treatment.

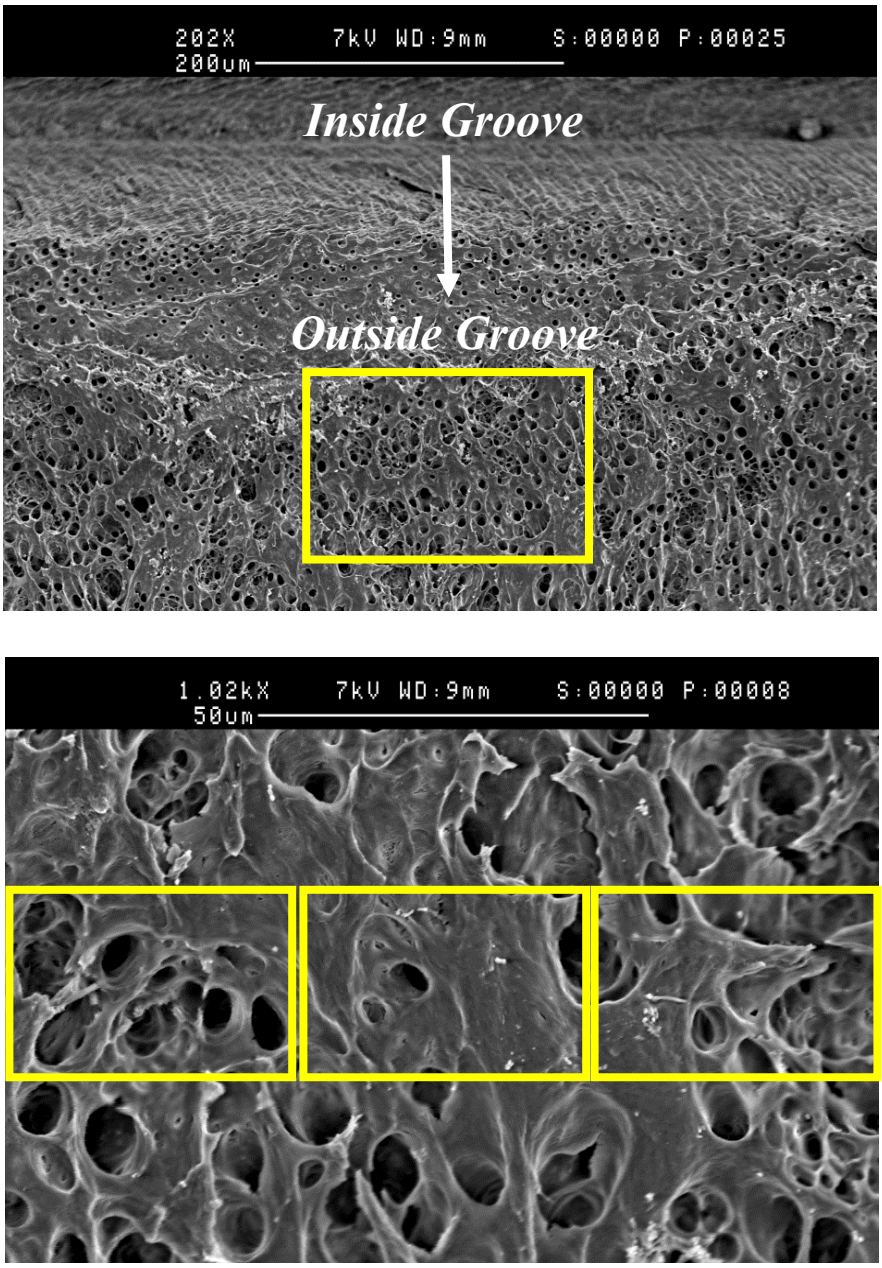

Figure 4.10 Stratified random sampling of SEM image. Using identical sampling methodology, SEM images from the areas outside the groove, adjacent to the sample sites inside the groove, were taken.

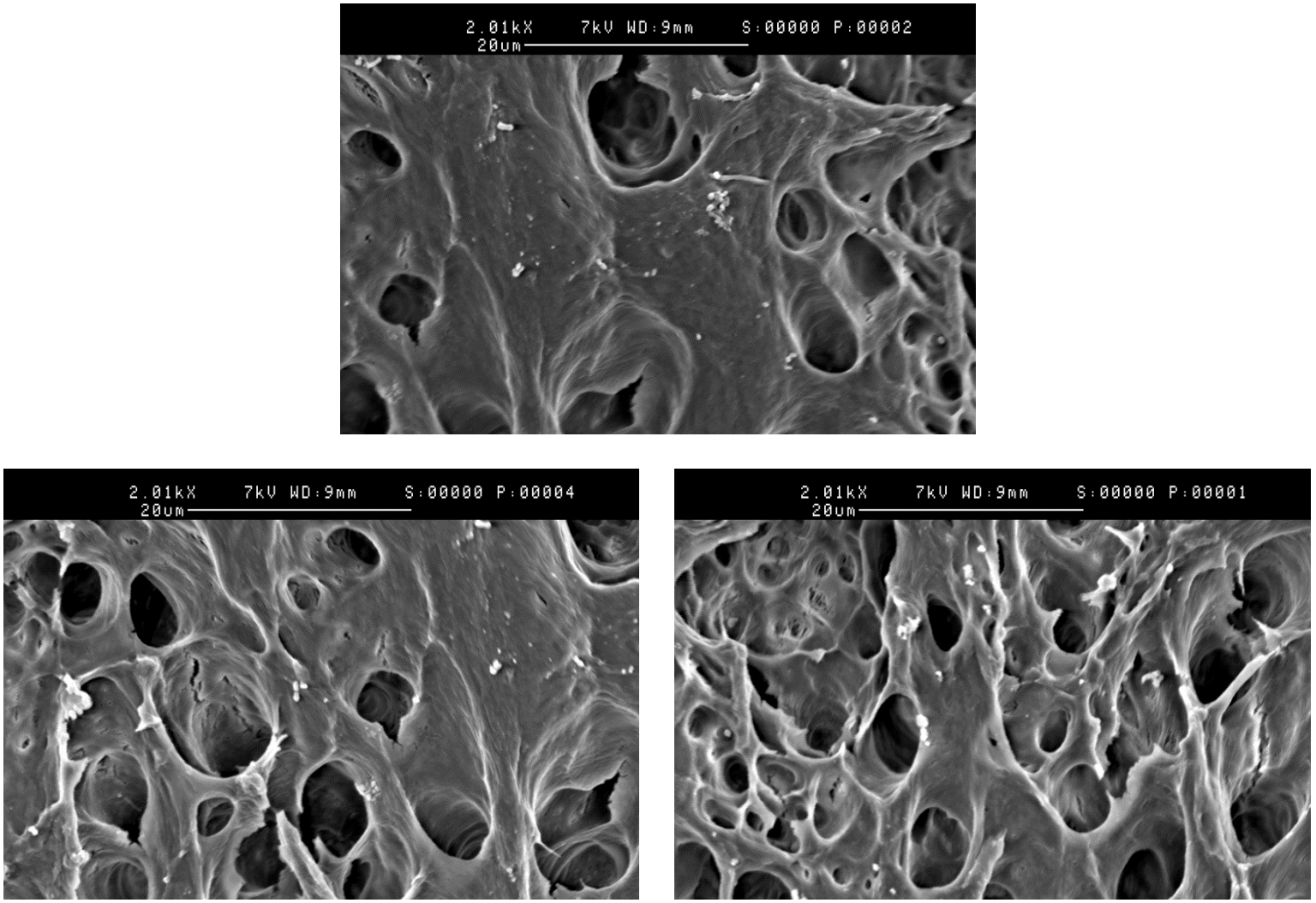

Figure 4.11 Standard SEM images at 2000 X obtained by stratified random sampling outside the groove for calculation of % bacteria remaining after treatment.

4.2.5 Evaluation of Biofilm Removal and Statistical Analysis

Standard SEM images taken inside and outside the groove for each treatment group were transferred to a computer and examined. The percentage area of biofilm bacteria remaining after treatment was determined using Image-Pro Plus Discovery 5.0 software (Media Cybernetics, Bethesda, MD). Bacteria measurement was accomplished semi-automatically using the software's auto-detection function (Figure 4.12). The examiner can only adjust the software's performance by discerning between the biofilm bacteria and artifacts in the digitized images. To ensure the reproducibility of the semiautomatic method, the SEM images were randomly coded and evaluated separately by two independent examiners. Inter-observer correlation was submitted for statistical analysis. The percentage area inside and outside the groove covered by biofilm bacteria after treatment by different groups were compared using the one way ANOVA and analyzed using SPSS (SPSS for Windows 11.0; SPSS, Chicago, IL).

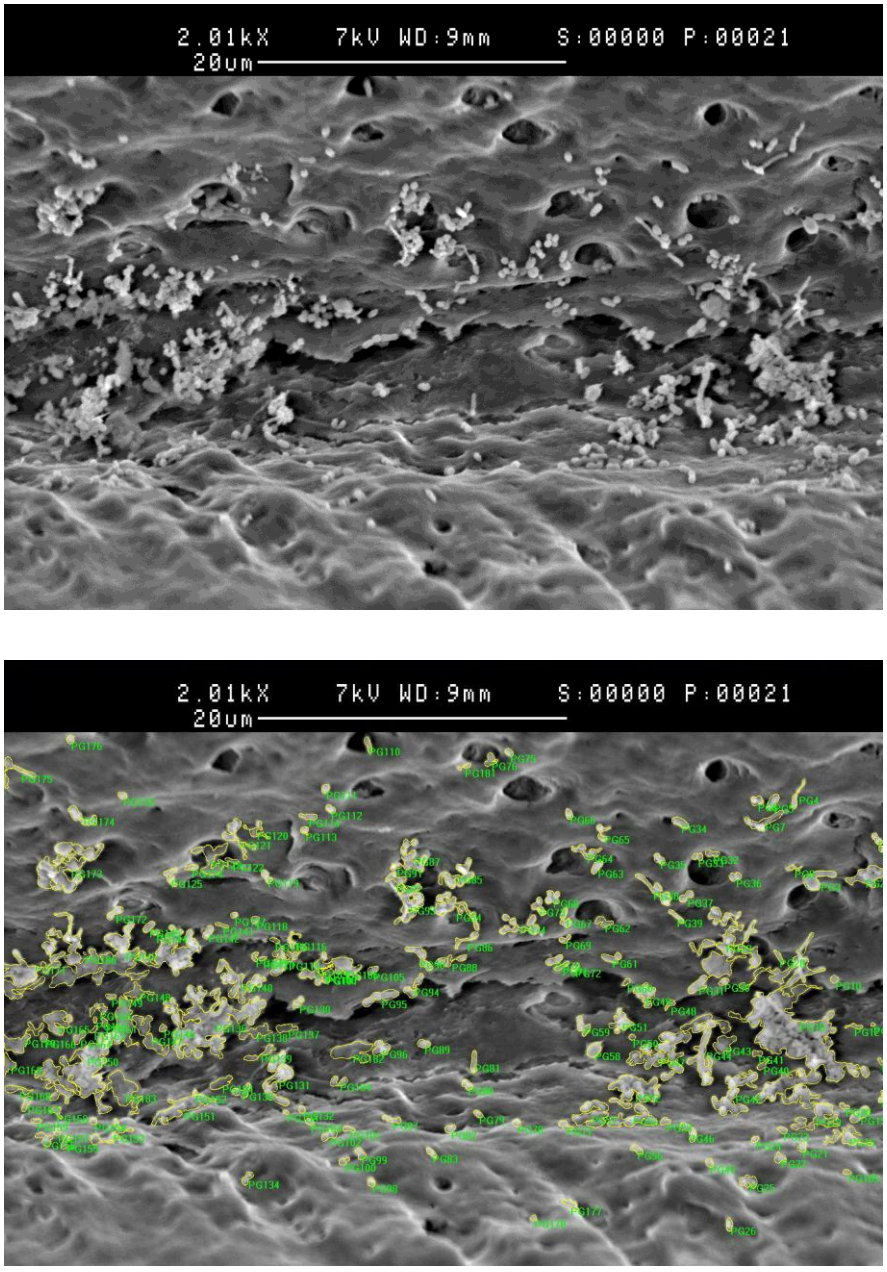

Figure 4.12 Measurement of percent area covered with bacteria. Standard image at 2000 X magnification (above) was used for determining the percent area covered with bacteria after treatment. Bacteria measurement was accomplished semi-automatically using Image-Pro Plus Discovery 5.0 software (below).

Chapter 5: Results

The inter-observer correlation coefficient was 0.91. Examination under SEM confirmed that all controls had grown a consistently thick layer of bacterial biofilm in the canal after 4 weeks (Figure 4.4, 4.5 and 4.6). SEM images showed the presence of multispecies, heterogeneous biofilms consisting of cocci, rods and filaments. Spirochetes were also found in high numbers throughout the biofilm surface (Figure 4.5 and 4.6). The biofilms grown within the canal of the extracted teeth were organized in network structures typical in appearance of natural biofilms. Within the groove, a smaller area remained occupied by bacteria after the use of SAF than ProFile and SS K-file (3.25%; 19.25%; and 26.98% respectively) ($P < .05$) (Figures 5.1, 5.2, and 5.3; Table 5.1). For all groups, significantly more bacteria were removed outside the groove than inside ($P < .05$) (Table 5.1). However, no statistical differences were found outside the groove between the treatment modalities (Figures 5.4, 5.5, and 5.6; Table 5.1). Within the same treatment group, no differences were found in the apical, middle and coronal regions (Table 5.1).

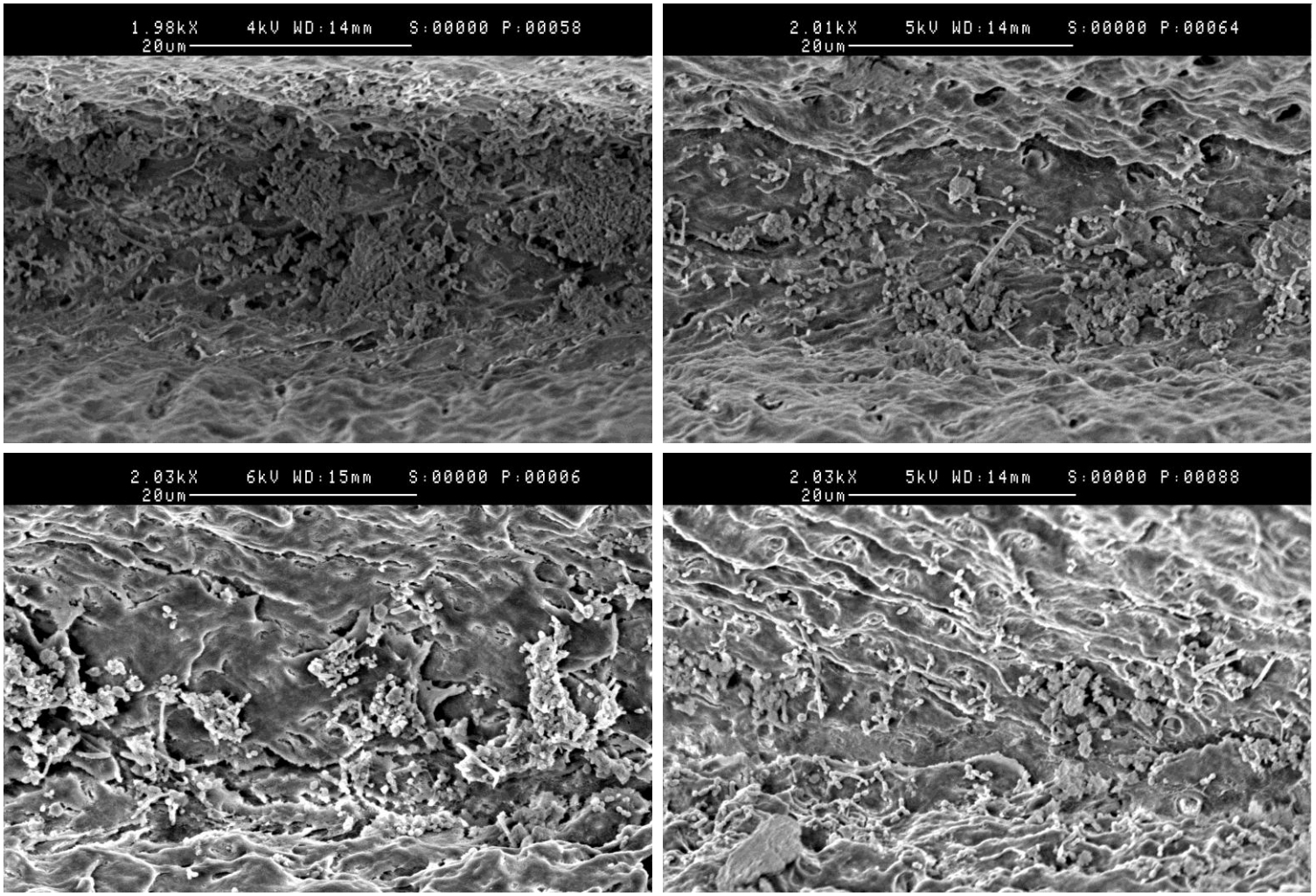

Figure 5.1 Treatment result *inside* the groove using SS K-file instrumentation and manual dynamic irrigation. Percent area remained covered with bacteria after treatment, 26.98 ± 4.66 %.

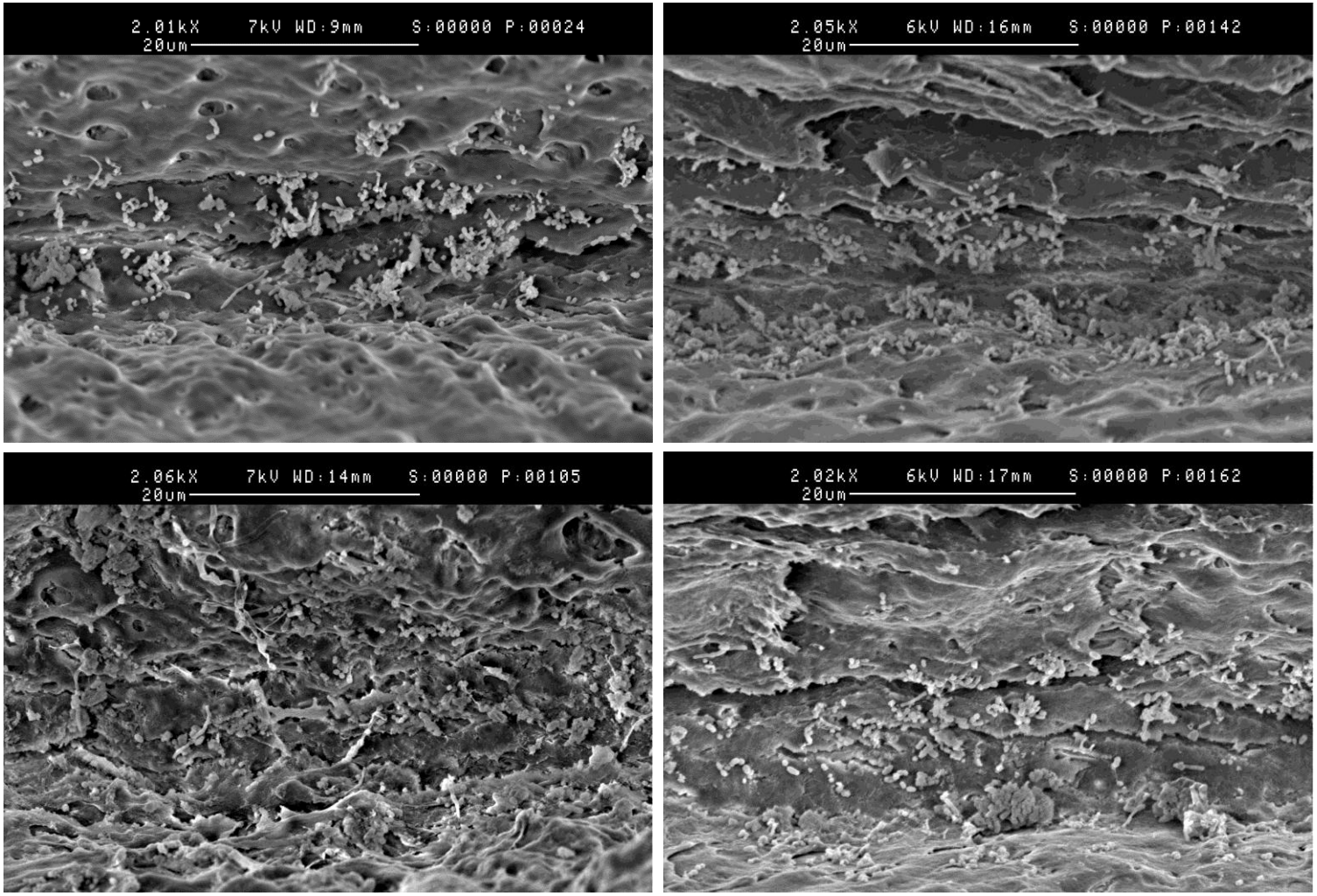

Figure 5.2 Treatment result *inside* the groove using ProFile NiTi rotary instrumentation and manual dynamic irrigation. Percent area remained covered with bacteria after treatment, 19.25 ± 4.29 %.

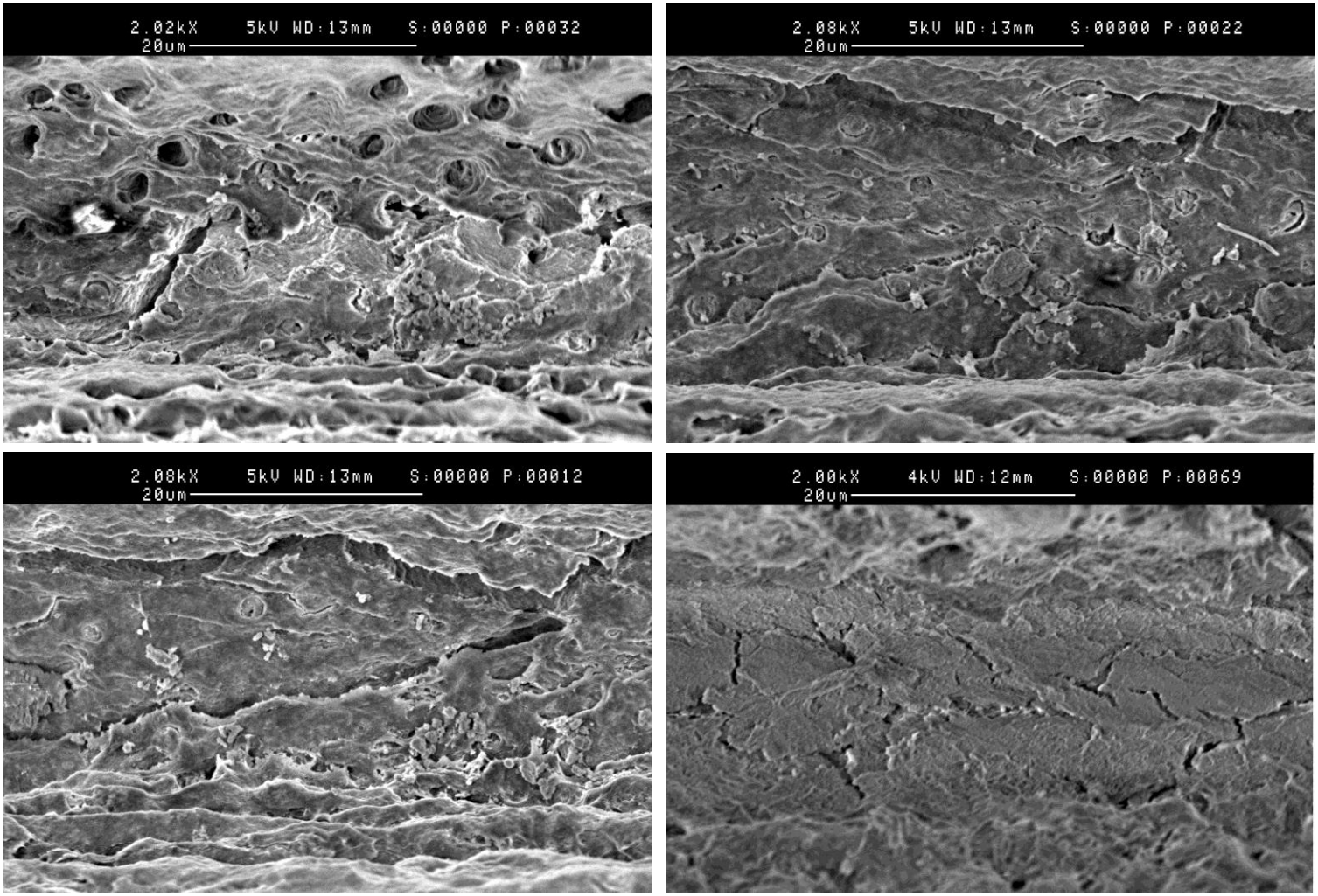

Figure 5.3 Treatment result *inside* the groove using SAF system and continuous irrigation. Percent area remained covered with bacteria after treatment, 3.25 ± 1.06 %.

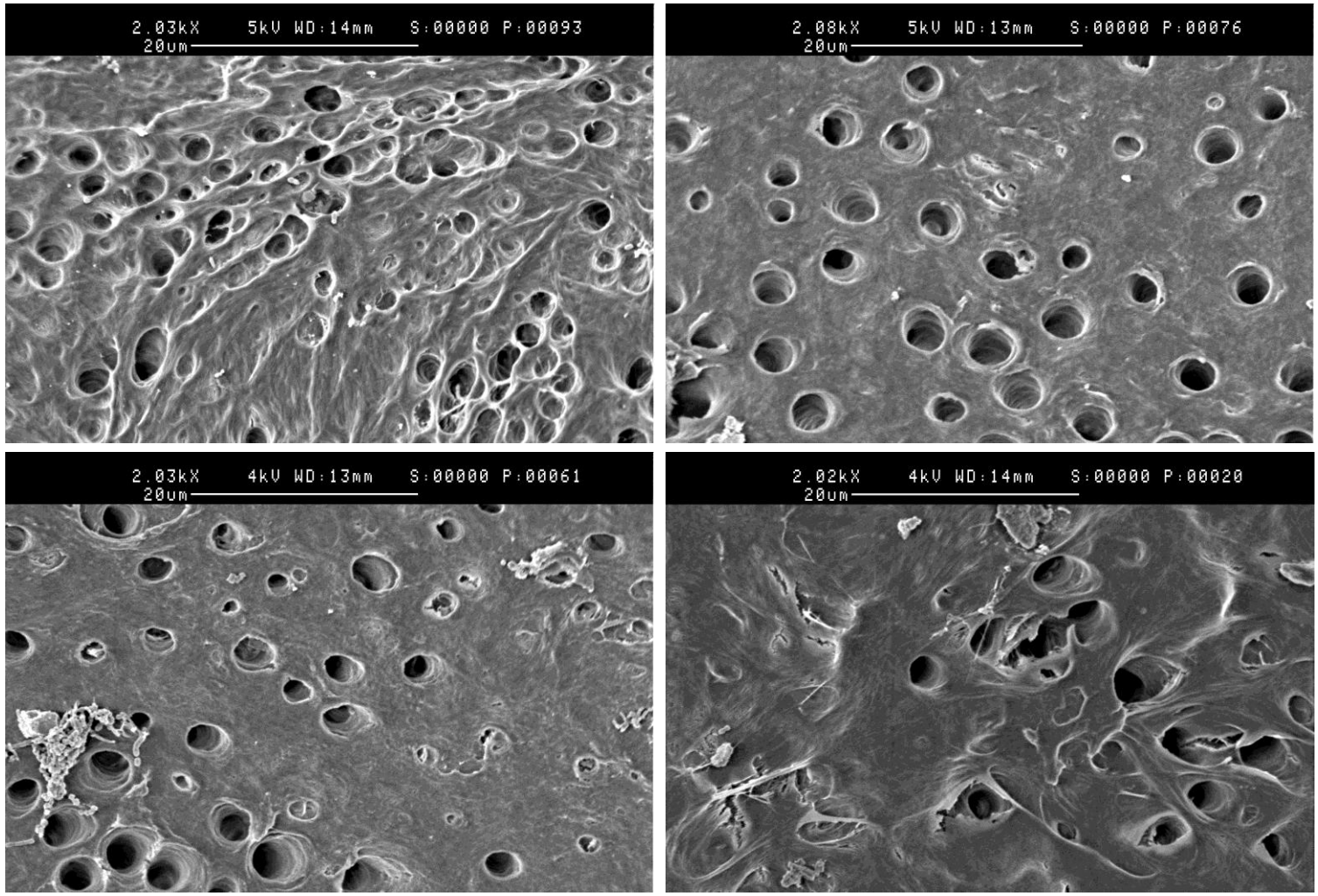

Figure 5.4 Treatment result *outside* the groove using SS K-file instrumentation and manual dynamic irrigation. Percent area remained covered with bacteria after treatment, 1.01 ± 0.90 %.

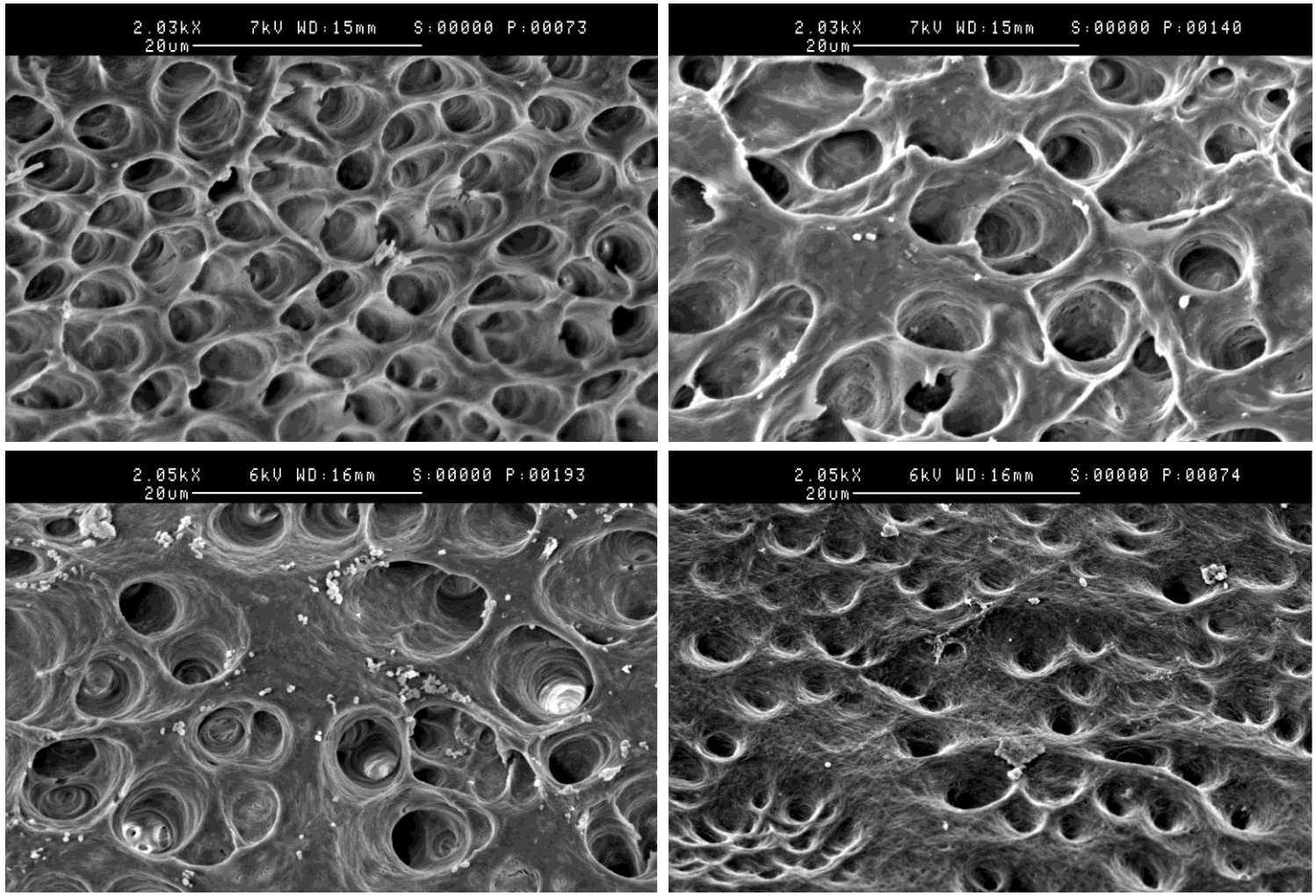

Figure 5.5 Treatment result *outside* the groove using ProFile NiTi rotary instrumentation and manual dynamic irrigation. Percent area remained covered with bacteria after treatment, 1.05 ± 0.83 %.

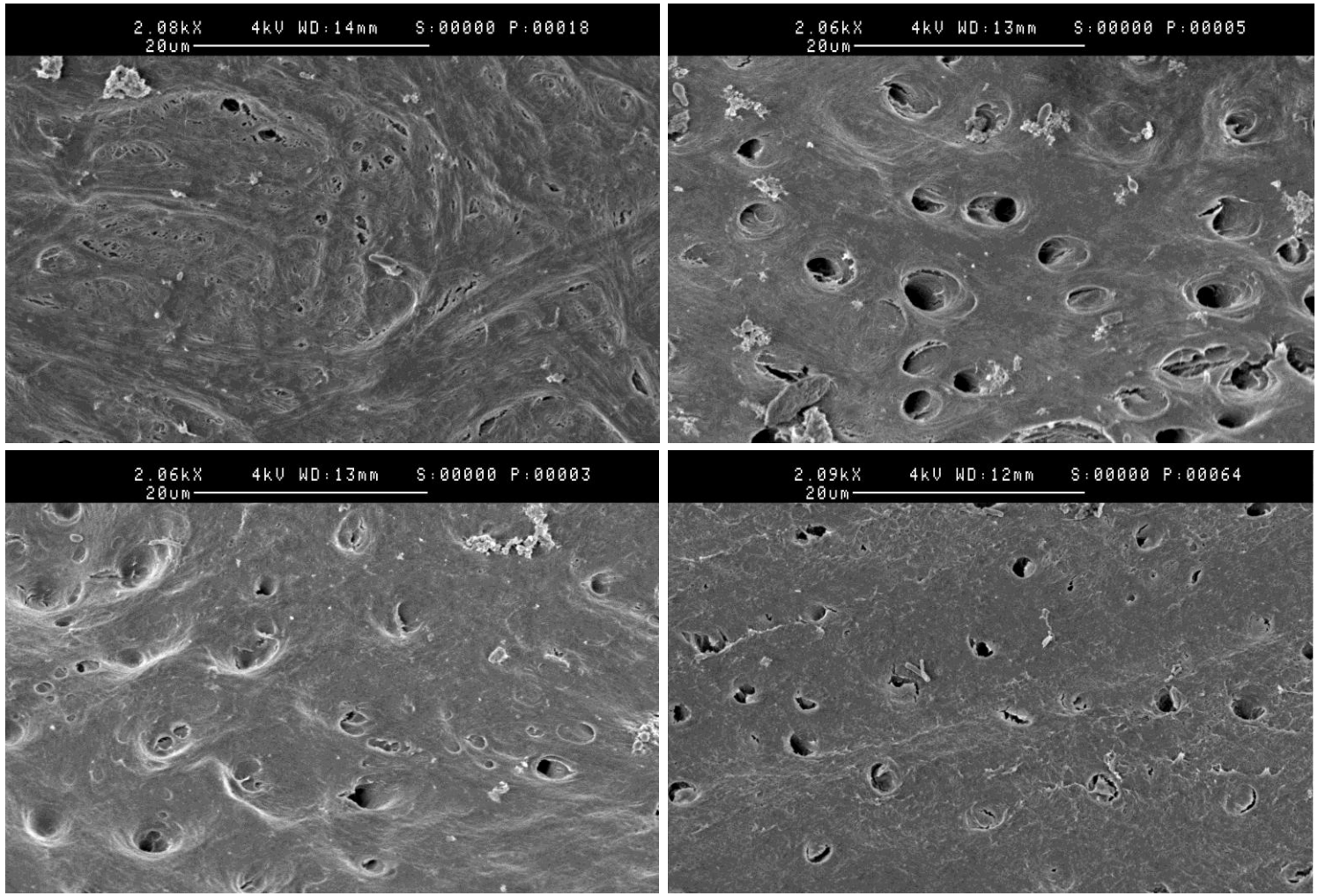

Figure 5.6 Treatment result *outside* the groove using SAF system continuous irrigation. Percent area remained covered with bacteria after treatment, 0.82 ± 1.03 %.

	Average % Area with Bacteria: *Inside* Groove (SD)				Average % Area with Bacteria: *Outside* Groove (SD)			
	Apical	**Middle**	**Coronal**	**Total**	**Apical**	**Middle**	**Coronal**	**Total**
Hand (SS K-file)	**28.29 (5.66)**	**26.49 (6.12)**	**26.16 (1.76)**	**26.98 (4.66)**	**1.26 (1.41)**	**0.84 (0.58)**	**0.94 (0.64)**	**1.01 (0.90)**
NiTi Rotary (ProFile)	**17.85 (4.39)**	**19.89 (4.38)**	**20.02 (4.72)**	**19.25 (4.29)**	**1.03 (0.88)**	**1.21 (0.84)**	**0.90 (0.92)**	**1.05 (0.83)**
SAF	**3.63 (0.74)**	**3.29 (1.20)**	**2.84 (1.25)**	**3.25 (1.06)**	**0.89 (0.92)**	**0.62 (0.43)**	**0.94 (1.62)**	**0.82 (1.03)**

Table 5.1 Average percent area of bacterial remaining from *inside* and *outside* the apical groove after treatment with hand SS K-file, ProFile NiTi rotary, and SAF (n = 10 per treatment group). Within the groove, the SAF group had significantly less biofilm bacteria remaining than the NiTi rotary and hand instrumentation groups (3.25% < 19.25% < 26.98) ($p < 0.5$). For all groups, significantly more bacteria were removed outside than inside the groove ($p < 0.5$). All techniques were equally effective in the bacteria removal outside the groove ($p > 0.5$). However, none of the techniques were able to completely remove the bacteria.

Chapter 6: Discussion

Endodontic diseases are polymicrobial infections in which the interactions between microorganisms play a significant role in determining the ecologic environment and the establishment of an endodontic habitat-specific multispecies microbiota. Intracanal microbial biofilms formed on the root canal dentin exhibit morphologically distinct types of bacteria. These biofilms display different bacteria-dentin wall relationships and distinct patterns of microbial organization. Given the unique environment of the root canal system, the development of realistic standardized mixed species anaerobic bacterial biofilms in extracted teeth is extremely difficult (Gulabivala *et al*. 2004).

In the present study, a standardized groove was placed in the apical part of the root canal simulating a fin into which multispecies bacteria consisting of cocci, rods, and spirochetes were grown. The apical region of the canal warrants special attention not only because disinfection of this area is the most difficult to achieve, but also because this region of the canal contains the highest incidence of anatomical irregularities that can harbour bacterial biofilms and is also in close communication with the surrounding periradicular tissues. The model described here provided a method for studying multispecies biofilms that closely resemble those present *in vivo*. The versatility of this model, combined with its simplicity and high reproducibility, makes it a potentially effective vehicle to study bacterial biofilm colonization and development on the root canal dentin. Since the biofilms found in teeth with established AP are likely to be more mature, with greater substrate adhesion and dentinal tubule penetration, the model presented in the current study attempts to replicate the heterogeneous nature of *in vivo* mature biofilms by allowing the bacterial community to grow for four weeks (Shen *et al*. 2011).

Microbiological root canal sampling is commonly employed to assess the effectiveness of endodontic treatment measures (Bystrom and Sundqvist 1981; Dalton *et al*. 1998). However, a limitation of all sampling techniques is that only the planktonic or free floating bacteria within the root canal system can be evaluated. Furthermore, bacteria which exist in a biofilm may assume a state of low-metabolic activity for the majority of time, similar to that of a stationary-phase planktonic state (Shen *et al*. 2011a). These bacteria in the low metabolic activity state may be undetectable by regular culture techniques. In addition, complex anatomical regions of the root canal system such as fins, accessory canals and isthmuses, may be inaccessible and therefore cannot be properly evaluated by microbiological sampling. An alternative way to study the efficacy of endodontic treatment in biofilm removal is to use SEM to directly observe intraradicular biofilms at a high magnification (Clegg *et al*. 2006; Kishen *et al*. 2006; Fimple *et al*. 2008; Bhuva *et al*. 2010). A criticism of SEM may be that only topographical assessment of the observed structures is possible. While this method cannot guarantee that the full depth of biofilm structures be observed, it compensates for the limitation by having the obvious advantages of using high magnification and allowing for observations to be undertaken from all regions of the possible sampling areas.

In order to reduce operator variability, a semi-automatic approach in measuring bacterial biofilms was applied in the present study. Standard SEM images were coded, and the percent area of biofilm bacteria was independently measured by two examiners. Professional imaging software was used to draw the biofilm outline automatically in high magnification, which eliminated the subjective assessment for the measurement. The operator manually controlled the software's performance only in distinguishing between

biofilm bacteria and other artifacts in the digitized images. This semi-automatic method provided operator-independent quantitative results and avoided the limitation of complete automated analysis technique, in which the software considered every single pixel on the whole image that passed beyond a threshold value. Using this method for quantifying bacterial biofilms, an inter-observer correlation coefficient of 0.91 was obtained.

Mechanical instrumentation is the main method for bacterial reduction in the infected root canal. With the advent of NiTi rotary systems, perhaps too much credit has been given to these systems as being the solution to overcome the challenges in root canal disinfection. Indeed, it is important to remember that no difference in the effectiveness of bacterial removal has been found between hand and rotary instruments (Siqueira *et al*. 1999). Dalton *et al*. (1998) sampled from root canals irrigated with saline solution before, during, and after instrumentation, then cultivated and counted colony-forming units. The investigators found that using instrumentation without antimicrobial irrigant, reduced the number of bacteria regardless of whether NiTi rotary or SS hand instrumentation was used. However, neither technique achieved bacteria-free canals. Their result was confirmed by Siqueira *et al*. (1999) showing that hand or rotary NiTi instrumentation combined with saline irrigation mechanically removed more than 90% of bacteria in the root canal. These findings lend support to our results here; namely, that hand and rotary files techniques are equally effective in bacteria removal in the main canal (outside the standard apical groove) yet are unable to achieve complete elimination. However, it appears that the rotary files are capable of achieving better biofilm bacteria removal within the apical groove than the hand files in our current study (19.25% versus 26.98% percent area bacteria remaining after treatment; $p < 0.5$).

It has been shown that the amount of mechanically prepared canal surface, and by extension a similar argument can be made with regards to biofilm removal, is dependent on geometry of the canal (Peters *et al*. 2001; Paque *et al*. 2009). Rotary NiTi instruments perform considerably poorer in long-oval canals such as the distal canals of the lower molars, as more than 60% of the canal surface remain untouched (Peters *et al*. 2001; Paque *et al*. 2009, Peters *et al*. 2001b). The bacterial biofilms on the uninstrumented surface are likely to remain mechanically undisturbed as well. A newly developed SAF has been designed to address the shortcomings of the traditional rotary files by having the capability of adapting itself to the canal cross section (Metzger *et al*. 2010a; Metzger *et al*. 2010c). As the compressible hollow NiTi tube adapts itself to the oval-shaped canal, its abrasive lattices are pressed against the walls promoting root canal enlargement due to the sandpaper-like effects. Comparing with rotary NiTi instrumentation, it has been reported that the SAF leaves less unprepared areas in anterior teeth (Peters *et al*. 2010; De-Deus *et al*. 2011) and molar root canals (Metzger *et al*. 2010a; Paque and Peters 2011). Furthermore, SAF system's hollow instrument design allows for simultaneous continuous irrigation during instrumentation to facilitate, debris and bacteria removal (Metzger *et al*. 2010a; Metzger *et al*. 2010c). Irrigants are purportedly delivered and exchanged in the apical region of the root canal as a result of the vibration and in-and-out motion of the SAF. Siqueira *et al*. (2010) compared the capability of SAF and rotary NiTi instrumentation to eliminate *E. faecalis* populations from extracted human teeth. Long oval canals from mandibular incisors and maxillary second premolars were infected with *E. faecalis* for 30 days to form biofilm-like structures. Preparation of long oval canals with the SAF was significantly more effective than rotary NiTi instrumentation in reducing intracanal *E. faecalis* counts. Data regarding the incidence

of negative and positive cultures revealed that whereas in the SAF group 80% of the samples were rendered free of detectable levels of *E. faecalis*, instrumentation with rotary NiTi instruments resulted in only 45% of culture-negative samples, indicating the SAF system has the potential to be particularly advantageous in promoting disinfection of oval-shaped canals. In the current study, bacteria residing in the canal fins or grooves make them inaccessible to the effects of conventional chemomechanical preparation. The study results appeared to have confirmed the superiority of SAF in bacterial biofilm reduction in these hard to reach apical anatomic irregularities. This may be related to the instrument's ability to contact a higher surface area of the canal walls, or the continuous delivery of fresh antibacterial irrigants throughout preparation, or both. Better instrumentation of the fins or grooves is also expected to provide improved access for irrigants to reach deeper within these areas, contributing to more effective elimination of the bacterial biofilms.

The standardized biofilm model represents a versatile and useful tool for studying root canal disinfection.

Chapter 7: Conclusion

The multispecies biofilm model using extracted single-rooted teeth with an artificial standardized groove in the apical canal, closely mimicking *in vivo* endodontic bacterial biofilm infections, served as a promising platform for evaluating the effectiveness of different techniques in biofilm bacteria removal. Although all techniques in the current study equally removed bacteria in the main canal outside the groove, SAF significantly reduced more bacteria inside the apical groove. No technique was able to completely remove the biofilm bacteria.

7.1 Future Directions

Using this model, various other methods of biofilm removal or killing utilizing negative pressure irrigation, the application of sonic and ultrasonic energy, or laser-activated disinfection, can be studied. In addition, future experimental design can include the use of confocal laser scanning microscopy to evaluate the efficacy of bacterial killing after treatment. The novel *ex vivo* biofilm model represents a potentially powerful tool for future study of root canal disinfection.

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Appendix

Appendix A: Informed Consent Form, Obtaining Donor Dental Plaque

Faculty of Dentistry
The University of British Columbia
www.dentistry.ubc.ca

Professor, Division of Endodontics
Markus Haapasalo, DDS, PhD
2199 Wesbrook Mall
Vancouver, BC Canada V6T 1Z3
Tel: [redacted] Fax: [redacted]
E-Mail: [redacted]

INFORMED CONSENT FORM: Test Subjects: dental plaque

"**Dynamics of killing biofilm bacteria**"

Principal Investigator: Markus Haapasalo DDS PhD [redacted]
Coinvestigators: Ya Shen DDS PhD, Sonja Stojicic DDS MSc, Zhejun Wang DDS
James Lin, DDS

Location: Room 328, Dentistry Bldg., UBC
24-hour emergency contact number: [redacted] (Dr. Markus Haapasalo)

1. Introduction:
You are being invited to take part in this research study because we require a study group of adult people who have teeth.

2. Your Participation is Voluntary:
Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this study. Before you decide, it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done, what will happen to you during the study and the possible benefits, risks and discomforts.

If you wish to participate, you will be asked to sign this form. If you do decide to take part in this study, you are still free to withdraw at any time and without providing a reason for your decision.

If you do not wish to participate, you do not have to provide a reason for your decision not to participate.

Please take time to read the following information carefully and to discuss it with your family, friends and/or dentist before you decide.

3. Who is conducting the study
The study is not receiving funds from an external agency or sponsors

4. Background Information:
Dental plaque is a biofilm, usually a pale yellow that develops naturally on the teeth. Like any biofilm, dental plaque is formed by colonizing bacteria trying to attach themselves to a smooth surface (of a tooth). Dental plaque can cause dental caries (tooth decay) - the localized destruction of the tissues of the tooth by acid produced from the bacterial degradation of fermentable sugars and periodontal problems such as gingivitis and chronic periodontitis. Further, bacteria from a caries lesion in the tooth crown can access to the root canal space (dental pulp) and cause infection in the root canal as well as in the bone surrounding the tip of the root.

Successful treatment of these diseases (dental caries, gingivitis, chronic periodontitis, root canal and periapical infections) is dependent on elimination of the microorganisms (bacteria). This is being done by brushing teeth with toothbrush and accessory products for dental hygiene and by cleaning the root canal with special instruments and irrigation (flushing) the canal space with solution which have antibacterial activity killing the microorganisms.

4. Purpose: Analysis of biofilms and factors involved in their ability to cause disease and evaluation of existing and development of new, more effective methods for their eradication from the root canal.
Bacteria, organized in biofilm structures, are the etiological factor of the common oral diseases such as caries, periodontal and root canal infections. Knowledge of the biofilm structure is important to understand its special characteristics as well as to find ways to eliminate it. Therefore, in this study we want to study biofilms in order to:

1) Develop *in vitro* biofilm models with close structural and functional similarity to *in vivo* biofilms and evaluate the effect of environmental factors such as availability and type of nutrients, oxygen, age and source of bacteria to their susceptibility to disinfecting agents.
2) To evaluate existing and develop novel disinfecting agents and/or protocols against oral bacteria for safe and more effective eradication of biofilms.

During regular cleaning of teeth with a tooth brush or a wooden stick you are removing plaque bacteria from your teeth. We would like to use these bacteria in our study in the laboratory.

5. Who can participate in the study?
Any adult person who has teeth.

6. Who should not participate in the study?
There are no medical contraindication (reasons not to participate) except for haemophilia (severe bleeding disorder).

7. **What does the study involve**?
In all subjects supragingival and subgingival dental plaque will be collected using the tip of wooden stick. Collected samples will be added to culture medium and used for biofilm growth on hydroxyapatite discs. Biofilms of different age will be later used for analysis of

factors important for their ability to cause disease and for testing and development of effective disinfection (treatment) methods.

8. If you decide to join the study:
If you agree to take part in this study, the procedures and visits you can expect will include the following:
One visit: Dental plaque will be collected with the tip of wooden stick from molars and premolars (teeth at the back of the mouth). The time required for taking the sample is 2 - 5 minutes.

9. Storage of Your Samples:
The samples of dental plaque collected for this study will be used immediately for biofilm growth in the UBC laboratory of Dr. M. Haapasalo. Unused samples will be destroyed. You may contact Dr. Markus Haapasalo [redacted] in order to ask questions. The results from your dental plaque samples will not be made available to you.

10. Benefits:
There is no direct benefit to you as a consequence of this study. Information gained in this study will aid in understanding of the causes and development of dental infections and help us improve our current therapies for these diseases.

11. Risks:
There are no additional risks or complications of the described sampling procedure.

12. What happens if I decide not to participate in the study?
Your participation in this research is entirely voluntary. If you decide not to enter the study, there will be no penalty and your future dental care will not be affected.

13. Who do I contact if I have questions about the study during my participation?
If you have any questions or desire further information about this study before or during participation, you can contact Dr. Markus Haapasalo [redacted].

14. Confidentiality:
Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, no records which identify you by name or initials will be allowed to leave the Investigators' offices. All documents will be identified by code number and kept in a locked cabinet. Should the results be published, individuals will not be identified in any way. Participants of the study will not receive any results.

Any concerns that you may have regarding your rights may be addressed by the Research Subject Information Line at the Office of Research Services. The phone number is (604) 822-8598. Your rights to privacy are also protected by the Freedom of Information and Protection of Privacy Act of British Columbia. This Act lays down rules for the collection, protection and retention of your personal information by public bodies, such as the

University of British Columbia and its affiliated teaching hospitals. Further details about this Act are available upon request.

15. Payment:
You will not be receiving any form of payment for participation in this study.

16. Participant Consent:
The study procedures have been explained to me completely. I understand that participation in this study is entirely voluntary and that I may refuse to participate or I may withdraw from the study at any time without any consequences to my continuing dental care. I will receive a signed and dated copy of this consent form for my own records.

After reading and understanding the above information, I hereby voluntarily consent to participate in this study.

Subject name (printed):_____

Subject Signature: _____ Date: _____

Principal Investigator or / designated representative name (printed):_____

Principal Investigator's
or designated representative's Signature: _____ Date: _____