CLINICAL AND MOLECULAR ANALYSIS OF SEALED CARIOUS DENTIN

Tijen Lacin, D.D.S.

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Horace H. Rackham School of Graduate Studies
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Thesis Committee Members:

Mathilde C. Peters, DMD, Ph.D. (Chair)
J. Christopher Fenno, Ph.D
Brian H. Clarkson, B.Ch.D., L.D.S., Ph.D., M.S.
Joseph B. Dennison, D.D.S., MS.
Peter Yaman, D.D.S., MS.

CONTENTS

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LIST OF TABLES

LIST OF FIGURES

Chapter I

1.1 Background and Significance

> *"The complete divorcement of dental practice from studies of the pathology of dental caries, that existed in the past, is an anomaly in science that should not continue. It has the apparent tendency to make dentists mechanics only."*
>
> *Green Vardiman Black, 1908* [1]

In almost every population studied, carious lesions have been found from the Australopithecines (over a million years ago) to the Neolithic (since 10,000 years ago). Caries, however, was very uncommon amongst fossil hominids into the Paleolithic and Mesolithic. There is a report of a jaw specimen of an early hominid dating from the skeletal record 200-500,000 years ago that had carious lesion on 11 of 13 maxillary teeth (specimen BMNH 686, from Broken Hill Mine, Zambia). In the most ancient hominids, the incidence of caries is less than 1%. Although many Neandertal specimens have been discovered, no carious lesions have been described except for a single root lesion in some Neandertal teeth from Mt. Carmel, Israel [2].

There has been sporadic, but generally increasing caries prevalence over the past 5,000 years. During the first 4,000 years there is a gradual increase in caries prevalence ranging from 2 to 10 carious teeth per 100 teeth, followed by a sharp rise at about the year 1000 A.D. to 24 carious teeth per 100 for 3 out of 4 populations. The year 1000 A.D. is the approximate date for introduction of sugar cane to the Western world (Figure 1.1) [3].

The earliest reference to tooth decay comes from the ancient Sumerian text discovered on a clay tablet known as the "Legend of the Worm" (Figure 1.2). Medical historians in ancient India, Egypt, Japan, and China also make reference to a "worm" as the cause of toothache. The Egyptians were the most advanced of the ancient civilizations prior to the Greece – Roman age. They were more concerned with treatment and placing fillings to treat the caries [4].

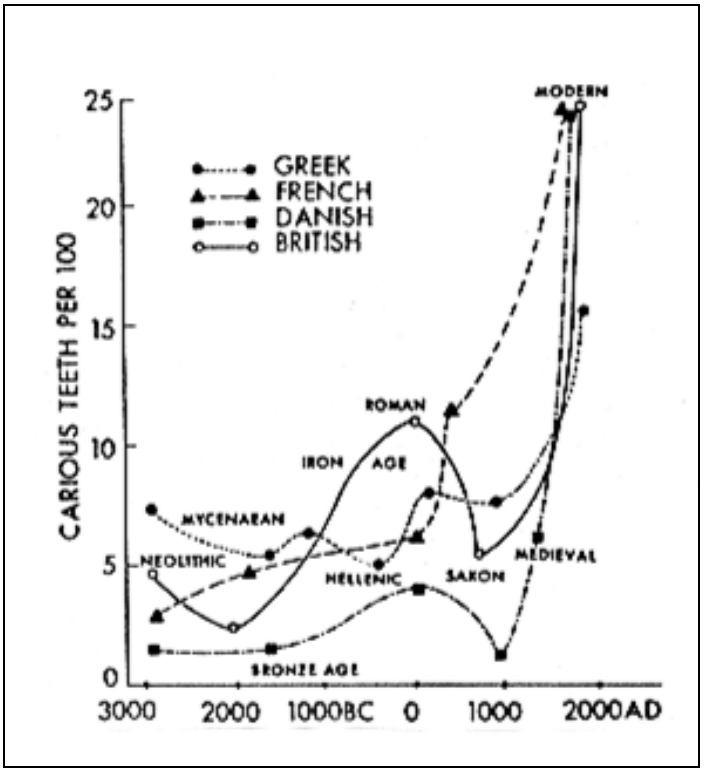

Figure 1.1 - Number of carious teeth per 100 teeth in four European populations

The incidence of dental caries dramatically increased from middle ages to 1950s. Dental caries has reached epidemic proportions affecting 90% to 95% of the population in the developed world by 1950s. From the early 1960s to the late 1970s fluoride research took place in the United States and the other parts of the world quietly.

Fluoride containing toothpaste offered significant additional protection. Since 1960s there has been a dramatic decline in caries incidence in most of the developed world [5-7].

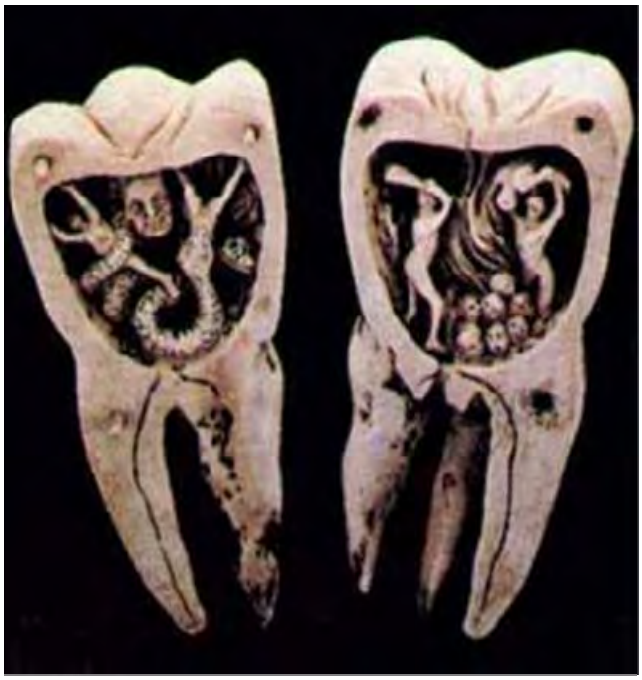

Figure 1.2 - The tooth worm: Babylonian tablet (about 7000 years ago) [2]

About 20 years ago, the internationally acclaimed decline in dental caries was thought to be an excellent example of the dental profession's success in caries prevention. Based on the decline in caries even authoritative institutes such as NIDR (National Institute of Dental Research) were giving off statements like "we are seeing the end of the disease" [8].

1.1.1 Epidemiology

The incidence of caries declined, but definitely it was not the end of the disease. Today dental caries is the single most common chronic childhood disease, 5 times more common than asthma and 7 times more common than hay fever. Over 50% of 5-9 year

old children have at least one filling or cavity and that proportion increases to 78% amongst the 17 year old population. In adults 18 and older, evidence of past or present coronal carious lesions was found in 94 percent of the population (NIDCR, 2002) [9].

The National Health and Nutrition Examination Survey (NHANES) has been an important source of information on oral health and dental care since 1970s. NHANES 1999-2004 oral health data represents the most recent cross-sectional information from which estimates can be generalized to the US population. Dye *et al.*, reviewed trends in oral health status that have occurred between the NHANES survey years 1988-1994 and 1999-2004. According to this survey dental caries continues to decline in the permanent dentition for youths, adolescents, and most adults since 1988-1994 (Figure 1.3). For adolescents and youths dental sealants prevalence has increased and dental caries has decreased. However, for youth aged from 2 to 5 years dental caries in primary teeth has increased [10].

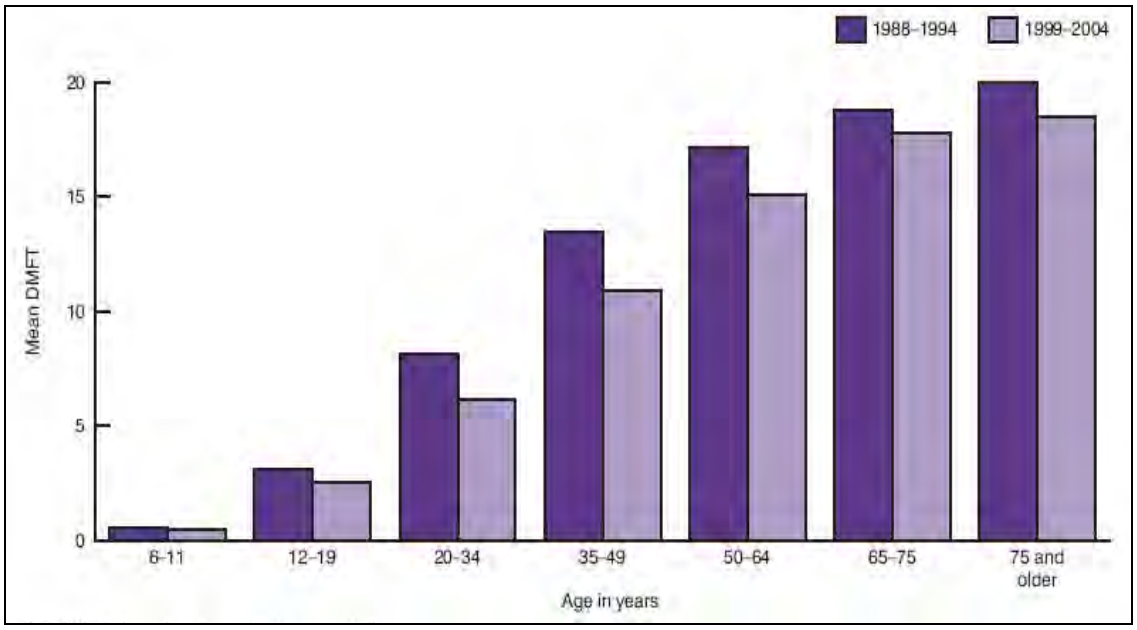

Figure 1.3 - Mean DMFT scores for persons 6 years of age and older by selected age groups: United States 1988-1994 and 1999-2004 [10]

The social impact of dental disease is substantial. More than 51 million school hours are lost each year to dental related illnesses. Poor children suffer 12 times more restricted activity days than children from higher income families. Pain and suffering due to untreated dental disease can lead to problems eating, speaking, attention to learning and even death (death of a 12 year old from Maryland, 2007) due to dental infection caused by untreated dental caries). Employed adults lose more than 164 million hours each year due to dental disease [11].

Expenditures for dental services alone made up 4.7% of the nation's health expenditures in 1998; $53.8 billion out of $1.1 trillion. Approximately 40% of charges are related to restorative dental services, which are usually performed to repair teeth damaged by carious lesions. During 1994, 2.9 million acute dental conditions occurred in the U.S. population. These dental conditions accounted for an estimated 3.9 million days of missed work in persons 18 years of age and over, 1.2 million days of missed school in youth 5 to 17 years of age, and 12.2 million days of restricted activity across all ages. These data show the impact of the disease on the American society [12].

1.1.2 Definition

Dental caries is described as a dynamic process taking place in the microbial deposits in dental plaque on the tooth surface which results in a disturbance of balance between tooth substance and surrounding plaque fluid so that, over time, the net result is a loss of mineral from tooth [13]. In simplistic terms dental caries can be considered as a victory of demineralization over remineralization at the localized tooth site [14].

1.1.3 Etiology

Miller and Black were two of the earliest educated dentists in the world. Miller might have been the first dentist who had thorough training in natural sciences and who was interested in understanding the biological basis of the dental caries process in 1879. He performed extensive studies of the oral microflora and its relationship to dental caries. The key discovery by Pasteur that microorganisms convert sucrose to organic acid helped Miller to develop a "catchy" term – the chemoparasitic theory.

The juncture of Miller's chemoparasitic theory with the description of dental plaque by Black provided key elements for the modern concept of etiology of dental caries. This concept has stimulated considerable scientific investigation and as a result it has become one of the essential paradigms of oral biology.

Even though the link between the microbial acid production from dietary substrates and etiology of dental decay was provided in the 19th century, researchers were not able to associate any single acidogenic species with decay. In 1960's, Loesche claimed that the mouth is colonized by 200 to 300 bacterial species, but only a limited number of these species participate in dental caries. In 1924 *Streptococcus mutans* was isolated from human carious lesions by Clark, but was not thoroughly studied until the 1960s. Loesche identified that *S. mutans* was the main cause of dental decay and various lactobacilli were also associated with progression of the lesion.

The factors involved in the caries process, which include a susceptible host, a cariogenic oral microflora and fermentable carbohydrate substrate, were first presented in the 1960's by Keyes (Figure 1.4). Each of these factors must be acting simultaneously for caries to occur. Since then the model was supplemented with the factors of time, fluoride, saliva and social and demographic factors (Figure 1.5). A

modern concept of caries also includes the importance of social, behavioral, and psychological factors as well as biological factors [17].

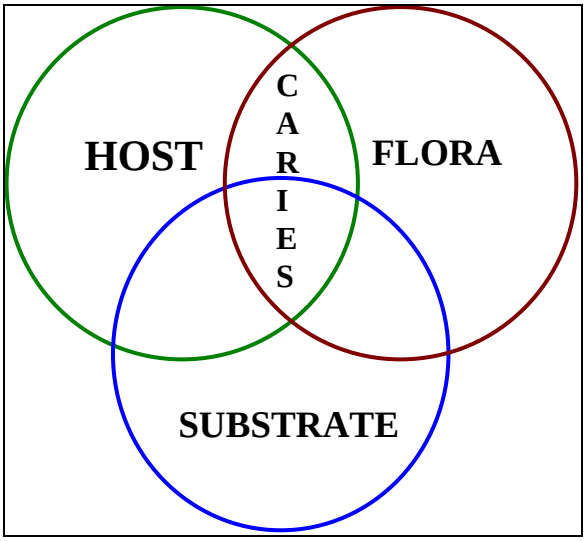

Figure 1.4 - Keyes' rings

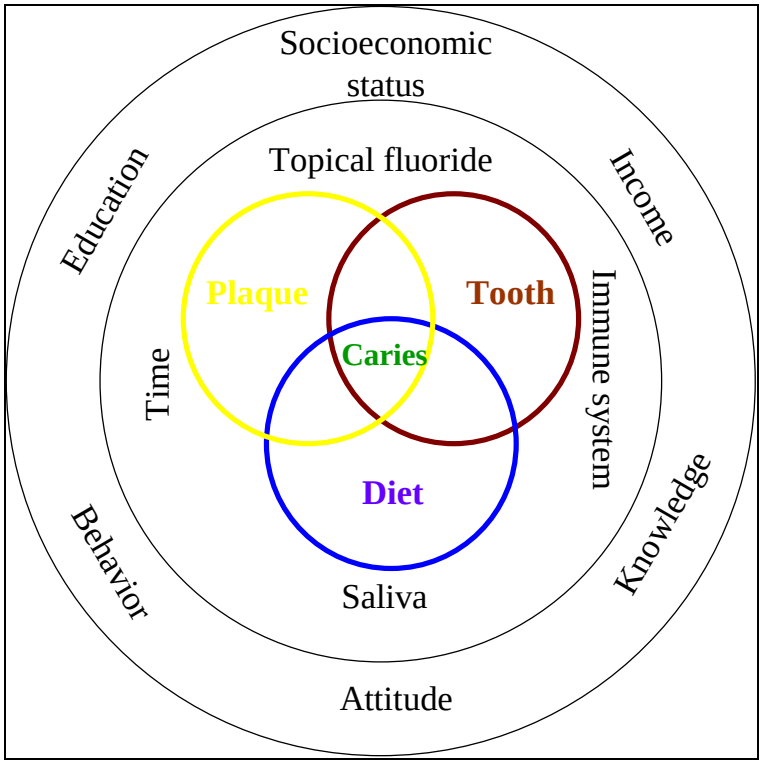

Figure 1.5 - Keyes' rings caries model supplemented with other risk factors (Summit et al., 2001).

S. mutans participates in the formation of biofilm on tooth surfaces. These biofilms are known as dental plaque. Sucrose is required for the accumulation of mutans streptococci. Also required for this accumulation are the enzymes glucosyltransferases (GTFs), which are constitutively synthesized by all *S. mutans.*

1.1.4 Dental Plaque

According to Miller's chemoparasitic theory, caries is brought about by acid dissolution of the mineral phase of the teeth, the acid being produced by metabolism of dietary carbohydrates by oral bacteria. In a secondary step, the organic phase of enamel and dentin is broken down. Considerable knowledge has been gained since the time of Miller's theory regarding the specific details of this complex disease.

The first event in the formation of dental plaque is the initial attachment of *S. mutans* to the tooth surfaces. The mutans streptococcal adhesin (known as antigen I/II) interacts with α -galactosides in the saliva-derived glycoprotein constituents of the tooth pellicle. Other components at the surface of *S. mutans* include glucan-binding protein (GBP), serotype carbohydrate and glucosyltransferases (GTFs).

In the presence of sucrose, GTFs synthesize extracellular glucans from glucose (after the breakdown of sucrose into glucose and fructose), and this is thought to be the second event in the formation of dental plaque. The mutans streptococcal protein GBP is a receptor-like protein that is distinct from GTFs, and it specifically binds glucans. GTFs themselves also have a glucan-binding domain and can therefore also function as receptors for glucans. So, *S. mutans* bind pre-formed glucans through GBP and GTFs, and this gives rise to aggregates of *S. mutans*. The metabolism of various saccharides

(including glucose and fructose) by the accumulated bacterial biofilm results in the production and secretion of considerable amounts of the metabolic end-product lactic acid, which can cause demineralization of the tooth structure when present in sufficient amounts in close proximity to the tooth surface. This is thought to be the third event in the formation of dental plaque, and it eventually results in a dental caries [18].

The metabolic activities in the oral biofilm covering a carious lesion are an assault on the pulpo-dentinal complex. In slowly progressing lesions, dentin is capable of mounting a defense through obturation of the tubules. This is caused by the physiological defense mechanism of sclerosis and by precipitation of mineral crystals within the tubules [19]. Both mechanisms lead to occluded, more mineralized, less permeable dentin [20]. Different pulpo-dentinal reaction patterns are to be expected based on the metabolic activities in the oral biofilm.

1.1.5 Caries Management

Ever since the dental profession became an independent medical field, the focus has been on the relief or elimination of pain caused by dental caries. Moreover, when treating the symptoms of dental caries, often, inadequate dental restorations placed at treatment of dental caries resulted in the need for replacement and further tooth damage over time. These restorations often resulted in the ultimate loss of teeth. The major reasons for removable and fixed prosthodontics and a variety of implants indirectly reflect results of the disease 'dental caries'.

Recently the emphasis of caries management has shifted from the traditional surgical model to controlling the disease, by promoting remineralization instead of solely removing demineralized tissue. Since the introduction of pit and fissures sealants

in clinical practice, dental professionals have been concerned that undetected small carious lesions could progress under a sealant.

It has long been hypothesized that sealing an existing carious lesion from contact with the oral fluids should lead to eventual reduction and even death of these organisms and, thereby, should arrest the lesion progression. Accordingly, the fate of the bacteria in carious lesions that are purposely sealed over has been of great interest to many researchers and clinicians.

Several studies have evaluated the arrest of carious lesions by sealing of the cavity following incomplete caries removal using cultural techniques to determine the bacterial quantity [22-30]. These studies have shown a quantitative reduction, or even elimination, of microorganisms, as well as clinical changes of dentin tissue to a harder and dryer consistency.

The actual activity of bacteria remaining under dental restorations and their ability to cause disease progression has not yet been assessed. If the remaining bacteria survive over time, they would require nutrients that might come from four possible sources: the oral environment/plaque (via marginal leakage of restorations), the pulp (via patent tubules/channels/porosities), other bacteria (via complex bacterial ecosystems in deep dentin lesions), and the degraded tissue in which they reside [31].

Mertz-Fairhurst *et al.,* 1998, provided long term, well controlled, clinical evidence that it was acceptable to leave demineralized dentin in the long term. The bonded and sealed composite restorations placed over frank cavitated lesions arrested the clinical progress of these lesions for up to 10 years [28].

All these studies attempted to determine activity of the bacteria under the restoration using culture methods. It has been shown that attempts to culture anaerobic bacteria from carious dentin result in a significant underestimation of the numbers of bacteria present [32]. Culture dependent methods for enumerating bacterial numbers are known to be biased since bacteria can only be cultivated if their metabolic and physiological requirements can be reproduced *in-vitro*. Where complex microbial communities are under investigation, such as the oral biofilm, enumerating bacteria by traditional microbial culturing techniques may produce erroneous results [33].

New molecular biology techniques have the potential to produce reliable means of quantifying bacterial DNA and therefore bacterial numbers. Molecular methods for bacterial identification and enumeration now make it possible to more precisely study the microbiota associated with dental caries. DNA sequence-based assays can be used to identify closely related species that are difficult to differentiate by traditional, culture-based approaches. In addition, 16S ribosomal DNA (rDNA) sequence-based clonal analysis allows for the detection and identification of species that are refractory to detection by traditional methods [34].

Polymerase Chain Reaction (PCR) based assays are capable of detecting the presence or absence of these disease-related bacteria with much greater sensitivity than culture [35]. The total number of anaerobes detected in carious dentin was 41-fold greater by real time PCR than colony counting [36]. PCR allows the specific amplification of target bacterial DNA in samples for which the background caused by other species is high [37]. Most importantly, in addition to quantifying numbers of bacteria, it should be possible to assess the metabolic activity of the bacteria.

From ancient times until the end of the 20th century epidemiological studies demonstrate that caries continues to be a public health problem world-wide. The complexity of dental caries is worthy of the attention of the finest scientific minds and application of the most sensitive techniques from epidemiology to molecular biology [21]. Although in the last few decades much progress has been made in understanding the caries process, risk factors and prevention, the problem of caries has carried over into the 21st century. To date, dental caries is still a wide-spread chronic disease and continues to be a major health problem in the world.

1.2 Purpose and Hypothesis

The purpose of this study is to compare clinical and microbiological changes in Class I caries lesions after incomplete carious dentin removal and ultraconservative tooth sealing by assessment at the following time periods: baseline and re-entry after 6 months.

Hypotheses:

H_O: Sealing of asymptomatic, dentinal caries (in the outer half of dentin) from the oral environment will have no effect in the reduction of the total bacterial load in the carious dentin and will not reduce activity of the remaining bacteria.

H_A: Sealing of asymptomatic, dentinal caries (in the outer half of dentin) from the oral environment will result in a reduction of the total bacterial load in the carious dentin and reduced activity of the remaining bacteria, leading to arrest of lesion progression.

Specific Aims:

1. To compare microbiological and clinical changes in carious lesions after 6 months of ultraconservative tooth sealing.

2. To evaluate the performance of molecular biological techniques to detect specific microbial populations in carious dentin both for quantity and metabolic activity.

3. To evaluate the clinical performance of bonded resin restorations over caries after 6 months.

1.3 Review of the Literature

The traditional management of carious lesions of any kind emphasized the removal of all infected and affected dentin to prevent further cariogenic activity and provide a well-mineralized base of dentin for restoration. Research has shown that it is not even possible to remove all bacteria from the carious lesions [38, 39]. These researchers used demineralized histological sections stained for bacteria to assess the persistence of bacteria after excavation. Shovelton found that approximately 40% of the examined teeth had some infected tubules on the pulpal floor and Crone found similar results on the basis of histological examination on 113 extracted teeth after complete excavation of deep caries lesions [38, 39]. Both authors stated that some bacteria were still persistent in many cavities prepared by classical techniques.

The cariologist opinion by Massler *et al.,* 1967 integrated a sophisticated and advanced biologic understanding of caries into caries treatment [40]. He pointed out that it was essential to differentiate active (acute) from the arrested (chronic) lesions on the basis of clinical criteria and histological descriptions. He stated that different pulpal reaction patterns were to be expected, requiring different means of treating caries as

opposed to only the radical approach. He also suggested that after removal of the infected dentin, effective sealing of the cavity would arrest the caries.

The traditional approach has been challenged in three main groups of studies which involved sealing soft caries into the tooth: (1) remove no caries and seal the decay (2) remove caries in stages over two visits, allowing the pulp time to produce reparative dentin (stepwise excavation) or (3) remove minimal caries at the initial access to a cavity and seal the remaining caries.

Group I - Handelman and colleagues have done extensive research on this subject. In their first study in 1976, they placed dental sealants on 60 teeth with carious lesions extending into the dentin [41]. Their control group included 29 unsealed teeth. They sampled teeth for bacterial culture at periods ranging from one week to two years. They found the greatest amount of bacterial reduction within two weeks after treatment. They found a substantial decrease in the number of cultivable microorganisms in sealed lesions when compared with the unsealed control teeth. Handelman's group, in a continuous study, describing a radiographic analysis of teeth treated similarly to their first study, reported a significant decrease in caries penetration in teeth in which the sealant remained intact [42].

Group II - Bjørndal and colleagues performed multiple studies on the procedure known as stepwise excavation. In this technique only the necrotic layer of dentin was removed at the first visit during the acute phase of caries progression followed by a temporary cavity seal to promote sclerosis of the dentin. The primary aim of the first excavation was to change the caries environment and not to remove as much carious dentin. In their 1997 study, they included 19 deep lesions and cultured bacteria from

dentin after the initial procedure that included peripheral dentin excavation and removal of the central cariogenic biomass and superficial necrotic dentin and after intervals of 6 to 12 months performing stepwise excavation. They also evaluated the clinical changes of dentin occurring during stepwise excavation [27].

They observed that colony forming units (CFUs) of the bacteria reduced substantially at re-entry. They also reported that dentin changes after the treatment interval were characterized by enhanced hardness of the dentin which was associated with marked reduction in bacterial growth. None of the study lesions resulted in pulp perforation during treatment. They concluded that initial removal of the cariogenic biomass appears to be essential for control of caries progression. They stated that stepwise excavation was not only an appropriate treatment of deep carious lesions but was also considered a suitable model for microbiological studies to determine the bacteria persisting in clinically excavated lesions.

Other than Bjørndal and colleagues many researchers have performed trials using the stepwise excavation technique. Two randomized controlled trials comparing stepwise and complete excavation are important for results, relevant to the risk of pulpal complications after complete removal of deep caries. In 1977, Magnusson *et al.* reported postoperative pulpal complications in 8 of 55 teeth (15%) treated by stepwise excavation and in 29 of 55 teeth (53%) treated by direct excavation [43]. Leksell *et al.*, 1996, similarly reported pulpal exposure in 10 of 57 teeth (17.5%) treated in stepwise technique compared with 28 of 70 teeth (40%) treated by direct excavation [44].

In his 2008 review on stepwise excavation, Bjørndal raised the question if the two-step excavation was necessary [45]. According to the survey by PEARL (Practitioners

Engaged in Applied Research and Learning), practice based research network, 18% of respondents would partially remove caries in a deep lesion and the vast majority of respondents would select an invasive approach, because they did not believe in leaving caries behind [46]. Only about one in five respondents said that they would choose to proceed with partial caries removal. The dentists who would use the invasive approach thought that a partial removal would fail sooner, and their patients would require endodontic treatment regardless of whether the respondents favored complete or partial caries removal and regardless of the respondent's restoration technique. These dentists also thought that if they left infected dentin, it might stimulate the obliteration of the root canals, making future endodontic treatment more difficult. Bjørndal, 2008, stated that this hypothesis would favor a second visit which would aim to assess the tooth's reaction and to remove the slowly progressing lesion in slightly infected discolored demineralized dentin before the final restoration [45].

Kidd, 2004, stated that stepwise excavation was an important evidence of the consequences of sealing infected dentin into teeth [47]. For this purpose they reviewed 13 stepwise excavation studies. The majority of these studies had no control group. The restorative materials for sealing the caries and times to re-entry were also varied. In these studies, caries activity was assessed clinically, radiographically and often by microbiological examination at initial entry or re-entry.

They concluded that:

1. The clinical success rate was high; symptoms rarely arose between excavations and exposure was usually avoided using stepwise excavation technique. Control lesions were often exposed by conventional excavation.

2. Dentin was altered on re-entry, being dryer, harder and darker in some studies.

3. Substantial reductions in cultivable bacteria were reported. In most studies some microorganisms survived. Two studies observed that the cultivable flora was altered on re-entry to a less cariogenic flora [29, 30].

4. There was a possibility that dental material used for restoration might have an effect on the outcome.

Kidd and colleagues mainly focused on the fate of the sealed bacteria under the restoration. They believed that the role of the biofilm in driving the caries process had some important clinical implications. If the biofilm is removed, partially or totally, mineral loss may be stopped or reversed toward mineral gain. In other words the lesion may be arrested and this can occur at any stage of lesion formation [49]. According to their understanding of caries, the caries lesion reflects the activity of the biofilm. The caries lesion is the result or symptom of the disease not the disease itself. Dentists and histologists focus on the reflection and accept the reflection as a disease forgetting the real action. The action is initiated in the biofilm [47]. Kidd *et al*., believe that current practice of caries removal took away much more than the bacterial plaque, and much enamel had to be removed to be able to inspect the softened, infected dentin.

Group III - Mertz-Fairhurst and colleagues used a randomized split-mouth, four-celled design to compare sealed composite restorations in teeth treated via no caries removal with both sealed and unsealed amalgam restorations in teeth from which all carious tissue had been removed [28]. The study population consisted of 123 patients aged from 8 to 52 years who had at least one pair of frank Class I lesions that, according to the investigator's radiographic evaluation, extended as far as halfway from the

dentinoenamel junction (DEJ) to the pulp. A total of 156 pairs (312 teeth) were included in the study. The investigators evaluated restorations radiographically as well as clinically at 6 months, 1 year and 2 years after treatment. They detected no significant differences among the three treatments, sealed conservative, sealed amalgam, unsealed amalgam, at any period. Mertz-Fairhurst's group followed up these patients for over 10 years, finally observing that "the bonded and sealed composite restorations placed over frank cavitated lesions arrested clinical progress of these lesions for 10 years."

Recent Systematic Reviews

Ricketts *et al.*, searched The Cochrane Oral Health Group Trials Register, Cochrane Central Register of Controlled Trials (Central), Medline, PubMed and Embase databases and published a valuable Cochrane Systematic Review in 2006 [50]. The selection criteria of the review were randomized controlled trials and controlled clinical trials comparing minimal (ultraconservative) caries removal with complete caries removal in unrestored permanent and deciduous teeth.

This electronic search identified 529 titles and abstracts out of which 49 full reports were obtained. They excluded 45 trials for the following reasons: no control, the ART technique compared to conventional caries removal where they judged the ART technique to constitute complete caries removal and the trials where a fissure sealant was placed over caries without any tooth preparation by comparison to conventional caries removal. They considered only four trials eligible according to their criteria for trial design, participants, interventions and outcomes [43, 44, 51, 52].

In the trials included in this review, 339 patients (604 teeth) were recruited into the trials and, of these, 538 teeth were available for analysis at the end of the studies. Where the results could be obtained the outcome measures were pulp exposure during caries removal, patient experience regarding pulpal inflammation or necrosis, progression of caries under the filling, and time until the filling was lost or replaced. They found evidence for the value of stepwise excavation as opposed to complete caries removal in the management of deep carious lesions in order to reduce the chance of pulpal exposure. They found no evidence that partial caries removal is deleterious in terms of sign and symptoms of pulpitis or necrosis in the immediate long term.

Based on their review they concluded that partial caries removal would appear to be preferable to complete caries removal in the deep lesions, to be able to reduce the pulp exposure risk. They also reported that there is insufficient evidence to know whether it is necessary to re-enter and excavate further in the stepwise excavation technique. Two of the four included studies did not re-enter and reported no adverse consequences [51, 52]. For the implication for practice they concluded that with only four included studies with a high risk of bias, and differences in lesion severity, firm conclusions could not be drawn and there was a need for continued research.

The PEARL researchers conducted a systematic search of five databases (Medline, Evidence-Based Medicine Reviews, the Cochrane Database of Systematic Reviews, Cochrane Central Register of Controlled Trials, and OVID's Database of Abstracts of Reviews of Effects) to identify studies relating to partial versus complete removal of carious lesions. They used “deep caries”, “deep carious lesions”, “partial caries removal”, “indirect pulp capping”, “pulpal exposure”, “stepwise excavation”,

"alternative restorative treatment (ART)" as key words. They limited their search to reports written in English describing studies using human subjects and published from 1950 through the first week of November 2007. The literature search yielded 1,059 articles, which the authors judged 23 to be directly relevant [46].

Their purpose was to bring an evidence-based approach to fundamental practice in general dentistry. They believed that the Cochrane article by Ricketts *et al.,* 2006, was extremely valuable, but it was limited in scope by virtue of being a meta-analysis focused only on the results of randomized controlled trials. Instead they sought to cast a wider net by performing a traditional review, taking into account observational studies and auxiliary investigations that also might be of interest to the practitioner.

As a result, they identified 10 articles accounting for 6 studies (four of these articles reported follow-up results) [51, 54, 28, 52, 55, 48, 56, 30, 57, 58]. The first three studies reported the results of randomized controlled trials. The authors concluded that after reviewing numerous clinical studies involving the use of partial removal or stepwise excavation to treat deep carious lesions, "there was substantial evidence that the removal of all infected dentin in deep carious lesions was not required for successful caries treatment" provided that the restoration could seal the lesion from the oral environment effectively. They also stated that additional clinical trials might be needed before this concept was accepted by the dental profession.

Griffin *et al*., 2008 searched electronic databases for comparative studies examining caries progression in sealed permanent teeth [59]. The purpose of this meta-analysis was to examine the effectiveness of dental sealants in preventing the progression of carious lesions in the pits and fissures of permanent teeth. In their search of MEDLINE using a

modified version of strategy used by NIH Caries Consensus Development Conference (University of Michigan 2003), (the MEDLINE search strategy was adapted to search EMBASE, the Cochrane Central Register of Controlled Trials), they identified 1905 records. After reviews by multiple investigators they included 6 studies including 4 randomized controlled trials (RCT) judged to be of fair quality, representing an estimated 384 persons, 840 teeth and 1090 surfaces [60, 61, 62, 63, 64, 65]. Four studies primarily sealed non-cavitated lesions, one exclusively sealed cavitated lesions, and one sealed both cavitated and non-cavitated lesions. Three studies used second or third generation resin-based sealants, two used glass-ionomer cement (GIC), and one used first generation resin-based sealants. Study populations included children, adolescents, and young adults ranging in age from 6 to 19 years.

They used random-effects model to estimate percentage reduction in the probability of caries progression in sealed versus unsealed carious teeth. (R= % Lesion Progressing SEALED / % Lesion Progressing UNSEALED). The median annual percentage of non-cavitated lesions progressing was 2.6% for sealed and 12.6% for unsealed carious teeth. The summary prevented fraction for RCT was 71.3% (95% CI: 52.8% – 82.5%, no heterogeneity) up to 5 years after placement. Despite variation amongst studies in design and conduct, sensitivity analysis found the effect to be consistent in size and direction. They concluded that sealing non-cavitated caries in permanent teeth was effective in reducing caries progression.

As a part of this meta-analysis Oong *et al.*, 2008, searched the same studies but focused on bacteria levels in sealed permanent teeth [66]. They used the same search strategy and results as described in the meta-analysis above. In order to measure the

effect of sealants on bacteria levels, they used the log^{10} reduction in mean total viable bacteria counts (VBC) between sealed and not-sealed caries and the percentage reduction in the proportion of samples with viable bacteria. Six studies including three randomized controlled trials, two controlled trials and one before-and-after study were included in the analysis [67, 41, 68, 69, 70, 71]. Although studies varied considerably, there were no findings of significant increases in bacteria under sealants. Sealing caries was associated with a 100-fold reduction in mean total VBC. Sealants reduced the probability of viable bacteria by about 50%.

As a result they found that sealants significantly reduced bacteria levels in cavitated lesions, but in some studies, low levels of bacteria persisted [66]. It appears that bacteria that persist under sealants cannot produce acid when isolated from the carbohydrate substrate and adequately sealed lesions are unlikely to progress. These findings support those of a recent meta-analysis, Griffin *et al.*, that sealants prevented caries progression. In combination, these two sets of findings suggest that when sealants are retained, and thus access to fermentable substrates is blocked, bacteria do not appear capable of exerting their cariogenic potential.

Microbiological Analysis

The microbial populations involved in dental caries are known to be highly complex and have not yet been fully identified, although key organisms to be associated with disease initiation and progression are generally recognized. The bacteria involved in caries initiation and early caries development, particularly the mutans group streptococci and lactobacilli, have been well-identified [72, 73]. As the lesion progresses, there is a

transition from predominantly facultative gram-positive bacteria in early caries to anaerobic gram-positive rods, cocci and gram-negative rods in deep carious lesions [74].

Many microbiological studies have attempted to determine which pathogens or combination of pathogens, initially residing in the surface biofilm, are responsible for dental caries [34]. Nearly all investigations into the microbial pathogenesis of caries have been conducted by cultivation of bacteria.

Past studies used culturing methods to detect and quantify the type and numbers of bacteria present within lesions. Attempts to culture anaerobic bacteria from carious dentin result in a significant underestimation of the numbers of bacteria present [32]. Culture dependent methods for enumerating bacterial numbers are known to be biased since bacteria can only be cultivated if their metabolic and physiological requirements can be reproduced *in-vitro*. These techniques may take several days to yield a result and therefore are inappropriate in situations where rapid diagnostic decisions are required. Where complex microbial communities are under investigation, such as the oral biofilm, enumerating bacteria by traditional microbial culturing techniques may also produce erroneous results [33].

New molecular techniques have the potential to produce reliable means of quantifying bacterial DNA and therefore bacterial numbers. Molecular methods for bacterial identification and enumeration now make it possible to more precisely study the microbiota associated with dental caries. DNA sequence-based assays can be used to identify closely related species that are difficult to differentiate by traditional, culture-based approaches. In addition, 16S ribosomal DNA (rDNA) sequence-based clonal

analysis allows for the detection and identification of species that are refractory to detection by traditional methods.

PCR based assays are capable of detecting the presence or absence of these disease-related bacteria with much greater sensitivity than culture [35]. PCR allows the specific amplification of target bacterial DNA in samples for which the background caused by other species is high [37].

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

PCR is a rapid, powerful method for generating unlimited copies of any target fragment of DNA, first described by Kary Mullis (1993). PCR has very quickly become an essential tool for improving human health and human life. The genetic material of each living organism plant or animal, bacterium or virus possesses sequences of its nucleotide building blocks (usually DNA, sometimes RNA) that are uniquely and specifically present only in its own species. Indeed, complex organisms such as human beings possess DNA sequences that are uniquely and specifically present only in particular individuals. These unique variations make it possible to trace genetic material back to its origin, identifying with precision at least what species of organism it came from, and often which particular member of that species.

Such an investigation requires, however, that enough of the DNA under study is available for analysis, which is where PCR comes in. PCR exploits the remarkable natural function of the enzymes known as polymerases. These enzymes are present in all living organisms, and their job is to copy genetic material, sometimes referred to as "molecular photocopying". PCR can be used to characterize, analyze, and synthesize

any specific piece of DNA or RNA. It works even on extremely complicated mixtures, seeking out, identifying, and duplicating a particular bit of genetic material from blood, hair, or tissue specimens, from microbes, animals, or plants, some of them many thousands or possibly even millions of years old.

Medical research and clinical medicine are profiting from PCR mainly in two areas: detection of infectious disease organisms, and detection of variations and mutations in genes, especially human genes. PCR can amplify unimaginably tiny amounts of DNA, even that from just one cell.

Most published PCR methods are not quantitative. This leaves them open to the criticism that they will fail to distinguish between major infections and those likely to have no clinical significance [35]. Several investigators tried to solve this problem by using real-time thermal cyclers, which allowed the rate of product formation to be quantified as the reaction proceeds, rather than simply obtaining data from the endpoint of the PCR reaction [75].

Recently, real-time PCR has been shown to be a sensitive rapid method for the detection and quantification of individual microbial species [75, 36, 32, 76]. Most real-time PCR tests use as a baseline the detection of bacterial small-subunit 16S rDNA or rRNA sequences. This sequence is expressed at essentially constant levels per cell, is highly conserved in all bacteria species yet contains species-specific sequences that permit detection of single species in complex microbial communities [76].

Real-time PCR is a cyclical enzymatic reaction in which two synthetic oligonucleotide primers and a fluorogenic probe hybridize to the nucleotide base sequences specific for the target nucleic acid sequence within a sample. As the PCR

reaction proceeds, the amount of fluorescence increases as the amount of PCR amplification products increase. By monitoring the release of fluorescence with each PCR cycle, the progress of the reaction can be recorded in real time and the amount of target DNA in the initial sample can be quantified, thus allowing the number of a given bacterial species to be enumerated [77].

The total number of anaerobes detected in carious dentin was 41-fold greater by real time PCR than colony counting [36]. In another study, *Porphyromonas gingivalis* was detected three times more often by PCR than by culture [38]. Results from initial experiments have shown this technique to be very effective in detecting the bacteria directly within samples of dentin excavated form varying levels through a lesion. Preliminary findings indicate that bacterial numbers in carious dentin fall away considerably at the advancing front of the lesion, but are still present to a greater degree than has been assumed. Real-time PCR results indicating bacterial numbers could be obtained in 6-8 hours from patient sampling, compared with 14 days for the colony counts. In addition real-time PCR reduces the chances of contamination, since it requires no additional handling of the PCR product [32].

One major advantage of this molecular technique is the fact that, in addition to quantifying numbers of bacteria, it is possible to assess the metabolic activity of the bacteria. In this method, instead of isolating total DNA from the cells, total RNA, including mRNA, is isolated. A “reverse transcription” is added to generate cDNA complementary to the mRNA target, and the resulting RNA/DNA hybrid molecule becomes the substrate for PCR. By this method it is possible to assess cellular activity by quantitating per cell mRNA levels of a target gene, because the mRNA is transcribed

in greater quantities when the cell is active [34]. In the present study quantitative real-time PCR technique was used to determine the initial bacterial load and the metabolic activity of bacteria in carious dentin after incomplete caries removal at baseline, and at time of re-entry.

Streptococcal cells are known to be quite resistant to standard lysis techniques. In order to obtain greater sensitivity different lysis methods were used in the literature. Most studies modified standard lysis technique with mutanolysin to maximize DNA yield and reported better results. Some studies, however, use "Bead Beater" lysis technique which requires extensive vortexing with very small glass beads [78]. Catt *et al.*, 2008, used mutanolysin for lysis of bacteria, using quantitative real-time PCR to examine differences in the plaque microflora from subjects who completed a one year longitudinal study on early childhood caries [79]. By detecting subtle differences in dental plaque they aimed to evaluate caries risk assessment. The results of this preliminary study suggested examination of plaque microflora as a tool for caries risk assessment. The present study used mutanolysin to maximize the bacterial lysis.

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Chapter II

2.1 Abstract

Background: The effect of sealing infected carious dentin on the surviving bacteria underneath dentin restoration has been subject of many studies. The culturing techniques used to determine bacterial count after incomplete caries removal showed quantitative reduction, or even elimination, of microorganisms. However, the number of studies using molecular techniques to detect the quantity and the activity of bacteria is minimal.

Purpose: The primary purpose of this study is to compare microbiological changes in sealed carious lesions in a 6 month period using molecular biology techniques. Secondary objectives include: **(1)** to evaluate clinical changes of carious dentin over a period of 6 months, **(2)** to demonstrate the added value of molecular biology techniques to detect the microbial population in carious dentin for both DNA and RNA.

Materials and Methods: Sixteen Class I carious lesions were minimally accessed under rubber dam isolation. Without any caries removal, dentin samples were collected and irreversibly infected dentin was sealed with bonded and sealed resin composite. After a 6 month time period the lesions were re-entered and clinical changes of dentin tissue were evaluated and compared to baseline data. Dentin samples were also collected at re-entry. Molecular biological techniques were performed on dentin samples to evaluate the quantity and metabolic activity of the total bacteria and *S. mutans* at baseline and 6 month re-entry.

Results: None of the teeth experienced clinical symptoms during the study period. No lesion progression was recorded radiographically. At 6 month re-entry carious dentin appeared harder, drier, and lighter in color. The application of an adhesive restorative system on irreversibly infected dentin did not affect the clinical performance of the restoration in the 6 month time period. No statistical differences were observed between baseline and re-entry in both the amount of total bacteria (DNA, $p=0.3714$), and the metabolic activity of total bacteria (RNA, $p=0.1551$). The observed decline in quantity of *S. mutans* proportion of total bacteria at re-entry compared to baseline was not statistically significant ($p=0.1158$). For the metabolic activity of the *S. mutans*, however, there was a statistically significant decline ($p=0.0165$) at re-entry compared to baseline.

Conclusions: Molecular biology techniques performed well to detect the microbial population in carious dentin both for quantity (DNA) and metabolic activity (RNA) of the bacteria. The major cariogenic bacteria, *S. mutans*, were not completely eliminated from the cavity but were significantly less active after 6 months of cavity sealing.

2.2 Introduction

The classic dental practitioner's approach to dental caries is removing infected dentin and filling the resulting cavity with a restorative material. However, cariologist's opinion is focused more on controlling the disease and remineralization therapy of carious dentin. Based on the current understanding of the disease, it is believed that dental caries reflects symptoms or results of ongoing and past disease. Drilling and filling dental caries will not possibly treat the disease itself. It has been stated that the cause of the carious lesion is not the bacteria inside the cavity but the bacteria in the dental plaque [1]. If the bacteria are in the cavity is sealed properly by restorative material, the nutritional path ways of the bacteria will be blocked and the bacteria will possibly die or become metabolically less active. Using this method it is possible to arrest caries progression.

Several studies have examined bacterial survival in incompletely excavated cavities after varying periods [2, 3, 4]. The common trends in these studies are the marked reductions in bacterial growth. There are no data to document that residual bacteria do any harm [5]. Microorganisms can adapt to dramatic changes and those left within the cavity can survive, presumably by deriving sufficient nutrients from tissue fluid in the pulp via dentin tubules, or perhaps from the tissue in which they remain [6].

Work by Mertz-Fairhurts *et al*., 1998, presented 10-year data on a series of occlusal restorations where they simply sealed off the carious lesions in dentin without removing the carious infected dentin [7]. They demonstrated that bonded and sealed restorations placed on soft carious dentin arrested the progress of these lesions and the restorations lasted in excess of 10 years.

Remineralization of remaining carious dentin was detected both biochemically and radiographically [8]. Tissue organization was shown through scanning electron microscopy. The results of this study indicated increased radiopacity in the first 6-7 months after sealing, no change in radiopacity between 6-7 and 36-45 months, decrease in radiolucent zone depth (subtraction radiography) and deposition of tertiary dentin. The partial removal of carious dentin and sealing of the cavity for a period of 36-45 months prompted pulpal defense reactions and arrested the caries process.

A scanning electron microscopic study by Parolo *et al.*, 2006, observed that there was no difference in the number of microorganisms per square millimeter in the active and inactive noncavitated lesions [9]. The fear of harmful bacterial action leads to a recommendation of complete caries removal of the contaminated tissue. The finding that arrested noncavitated lesions harbored bacteria showed that the presence of bacteria within the dental tissue would not necessarily lead to the need for an operative treatment.

Thompson *et al*., searched five electronic databases for clinical studies involving the use of partial removal or stepwise excavation to treat deep carious lesions. They concluded that there was substantial evidence that removing all vestiges of infected dentin from deep lesions was not required for caries management provided that the restoration could effectively seal the lesion from the oral environment [10].

Oong *et al*., (2008) searched electronic databases for comparative studies examining bacterial levels in sealed permanent teeth [11]. There were no findings of significant increase in bacteria under sealants. Sealants significantly reduced bacterial levels in cavitated lesions, but in some studies, low levels of bacteria persisted. These findings supported those of a recent meta-analysis by Griffin *et al*., (2008) that sealants prevented

caries progression [12]. These findings suggest that when sealants are retained, and thus when access to fermentable substrate is blocked, bacteria do not appear capable of exerting their cariogenic potential.

Past studies used culturing methods to detect and quantify the type and numbers of bacteria present within lesions. It has been shown that cultural techniques have some limitations. These techniques underestimate the amount of bacteria by 40% and they are known to be biased since bacteria can only be cultivated if their metabolic and physiologic requirements can be reproduced *in vitro* [13].

Molecular biology techniques have the potential to produce reliable means of quantifying bacterial DNA and therefore bacterial numbers. Molecular methods for bacterial identification and enumeration now make it possible to more precisely study the microbiota associated with dental caries. DNA sequence-based assays can be used to identify closely related species that are difficult to differentiate by traditional, culture-based approaches. In addition, 16S ribosomal DNA (rDNA) sequence-based clonal analysis allows for the detection and identification of species that are refractory to detection by traditional methods.

One major advantage of this molecular technique, Reverse-Transcriptase (RT) Polymerase Chain Reaction (PCR), is the fact that, in addition to quantifying numbers of bacteria, it is possible to assess the metabolic activity of the bacteria. In this method, instead of isolating total DNA from the cells, total RNA, including mRNA, is isolated. A “reverse transcription” is added to generate cDNA complementary to the mRNA target, and the resulting RNA/DNA hybrid molecule becomes the substrate for PCR. By this method it should be possible to assess cellular activity by quantifying per cell

mRNA levels of a target gene, because the mRNA is transcribed in greater quantities when the cell is active [14].

The primary purpose of this study is to compare microbiological changes in sealed carious lesions in a 6 month period using molecular biology techniques. Secondary objectives include: (1) to evaluate clinical changes of carious dentin over a period of 6 months (2) to demonstrate the added value of molecular biology techniques to detect the microbial population in carious dentin for both DNA and RNA.

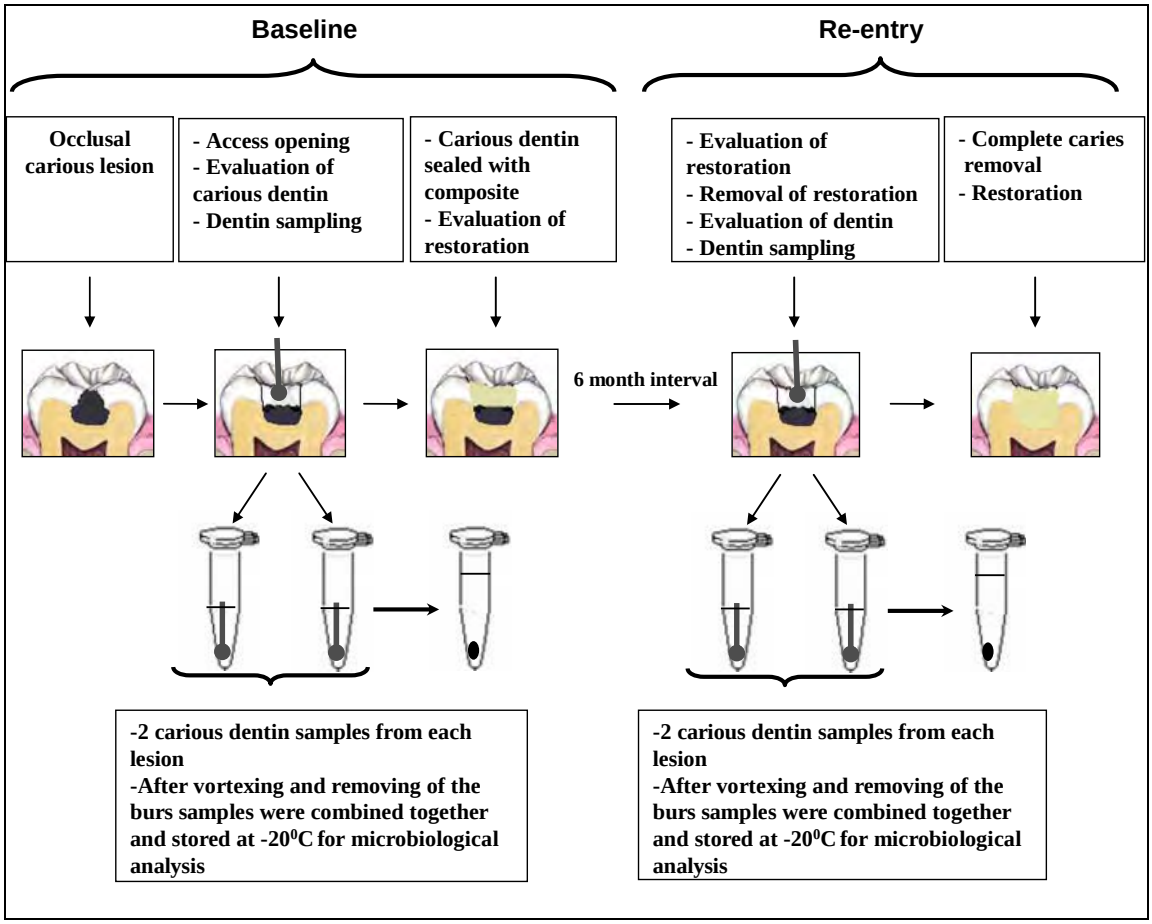

Figure 2.1 - Study design

2.3 Materials and Methods

2.3.1 General

Clinical Study Overview – A total of 19 carious lesions in 9 patients were included in this study. Prior to initiation of the study, approval for the project was obtained from the local institutional review board (The University of Michigan, I.R.B Health Sciences # 1336). All of the lesions in the study were identified in patients of record at the University of Michigan School of Dentistry, and recruitment was by referral from dental care providers in the Graduate General Dentistry Clinic. Once prospective patients were identified, the scope of the study was explained to them. Patients desiring to participate in the study were required to sign and date an informed consent form **(Appendix A).** At the end of the study the complete caries removal was obtained and cavities were restored with composite resin material. The restorations were placed at no charge and a small monetary incentive was given to the patients as an acknowledgement for their participation and time involved **(Appendix B).**

Inclusion Criteria – Patients in good health who had at least one permanent molar/premolar vital and non-symptomatic occlusal/cervical carious lesion and who were available for future recall attendance (6 months) were included in this study. The lesions were detectable on the radiograph. The occlusal and cervical carious lesions were diagnosed by the referring dentist based on existing clinical and/or radiographic findings and verified by the primary investigator (tl) and primary mentor (mp) prior to treatment.

Exclusion criteria – Patients who were medically compromised and/or allergic to methyl methacrylates were excluded from the study. Teeth with deep cavitations, pulpal symptoms, or approximal lesions were excluded. Primary teeth were excluded, and teeth

that were not possible to isolate with rubber dam were not eligible. Table 1 shows the location of the 19 lesions included in the study. Sixteen of the lesions were occlusal and 3 of the lesions were cervical lesions. Sixteen of the occlusal lesions were on 2 maxillary premolars, 2 mandibular premolars, 6 maxillary molars and 6 mandibular molars. The 3 cervical lesions were on 3 mandibular premolars. Of the patients who participated in the study, 4 were female and 5 were male, were all treated in the Graduate Dentistry clinic between January 2005 - August 2005.

Table 1 - Distribution of Lesions

	Class I	Class V	**Total**
Maxillary Premolars	2		2
Mandibular Premolars	2	3	5
Maxillary Molars	6		6
Mandibular Molars	6		6
Total	16	3	**19**

All of the restorative procedures at baseline and re-entry were performed by the primary investigator (tl) according to a standardized clinical protocol **(Appendix C).** Evaluations of the lesions were done by the primary investigator (tl) and primary mentor (mp), trained and calibrated in the assessment procedures through evaluation of a series of clinical lesions in student clinic patients prior to the study.

2.3.2 Clinical Procedures

2.3.2.1 Radiographic Protocol

Standard periapical and bitewing x-rays (F-speed, double pack films, Eastman Kodak Company) were taken of the occlusal lesions prior to the treatment using an x-ray holder (Figure 2.2). One of the films was used for the study and the duplicate was placed in the patient record. In order to standardize, all films were exposed by the

primary investigator (tl) using the same x-ray unit (Gendex GX-770, Gendex Corporation, Des Plaines, IL). Posted guidelines were used (70KVp, 7 Ma 11 Pulses) when exposing the films. Bitewing x-rays were taken using a customized holder to enable accurate duplication of the position of the x-ray cone at the 6 month re-entry appointment (Figure 2.3). Retentive holes were placed for the bite registration material on the holders. A vinyl polysiloxane bite registration material (Regisil, Dentsply Caulk) was used for the bite registration. Following placement of the registration material on both sides of the x-ray holders, patients were asked to bite on the holder. After removal from the mouth, excessive registration material was taken away with a Bard Parker surgical blade #15. After confirming the correct bite, the bite wing x-ray was taken. Each of the films was developed on the day it was exposed in an automatic roller-type processor (Gendex GXP Model 110-0096 G1, Gendex Corporation, Des Plaines, IL) with self-replenishing solutions (Supermax GX solutions, Gendex Corporation, Des Plaines, IL).

Figure 2.2 - Rinn XCP film holding system; holder for periapical x-rays (yellow) and bitewings (red)

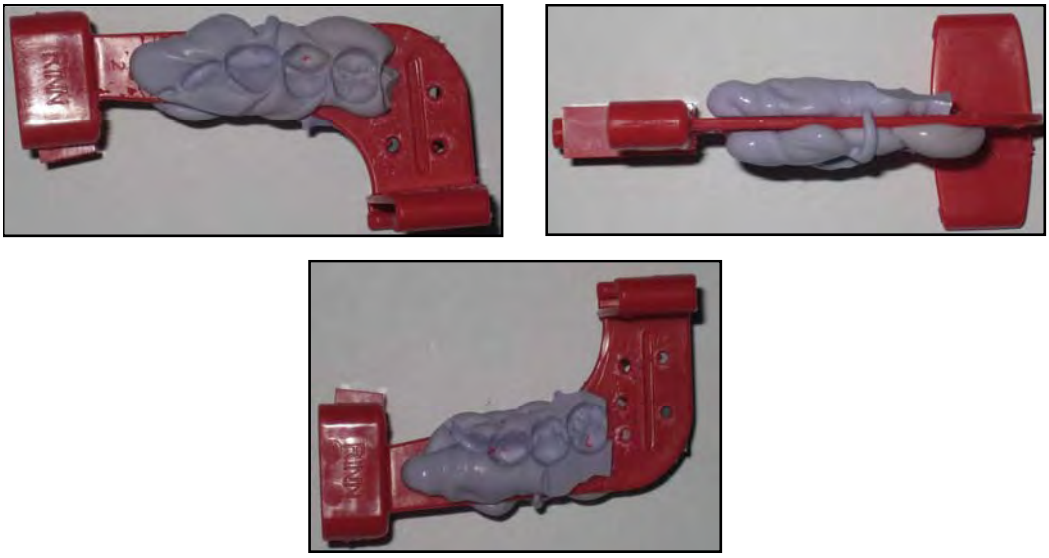

Figure 2.3 - Customized Rinn bite wing holder; maxillary, lateral, mandibular views

2.3.2.2 Clinical Protocol – Baseline

The clinical sequence form **(Appendix D)** was followed for each clinical procedure to standardize each appointment and to avoid missing steps. A clinical case report (CRF) / clinical evaluation form (CEF) was filled out at baseline **(Appendix E)** and at re-entry **(Appendix F)** appointments. The patient's medical history was reviewed before starting the clinical procedure. Depth of the demineralization was evaluated on the x-rays and noted either less than 25%, or 25-50% depth into dentin. Before beginning the clinical procedure, each patient was interviewed for tooth sensitivity. Patients were asked to scale sensitivity for the study tooth on a 0-10 scale (0: no sensitivity, 10: extreme sensitivity). Following the interview, the pulp vitality was noted using electrical pulp testing for both the study tooth and the contralateral tooth. DMFT (decayed, missing, and filled teeth) scores were also determined for each patient. All of the operative/restorative treatments and biopsies were performed by the primary

investigator (tl). Standard local anesthesia (2% Lidocaine HCl with 1:100.000 epinephrine) was performed as needed. The study tooth and adjacent teeth were cleaned with a black prophy brush by using water only. Tooth isolation was performed with a rubber dam including a minimum of three teeth. A chlorhexidine (CHX) swab (ORIS Chlorhexidine Gluconate Oral Rinse 0.12%, Dentsply Professional) was applied to the isolated teeth and the rubber dam. Extra care was taken so that the CHX swab was not applied on the cavitation surface. The pre-operative photograph (5X magnification) of the lesion was taken using a Nikon N6006 AF camera body (medical Nikkor lens 120mm, Tokyo, Japan). The fissures of the tooth were refreshed with high speed sterile #1/2 round bur. The initial enamel preparation was carried out with a sterile conventional high-speed hand piece using a sterile no. 330 carbide bur (1.7 mm length) (Figure 2.4**).**

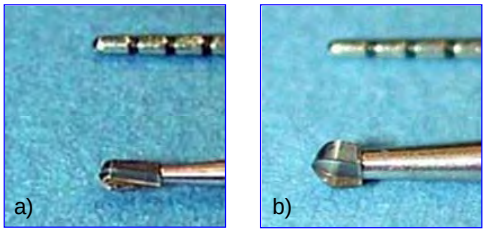

Figure 2.4 - a) # 330 bur along with perio probe to mark its length (1.7 mm); b) # 4 round bur (caries biopsy) with perio probe.

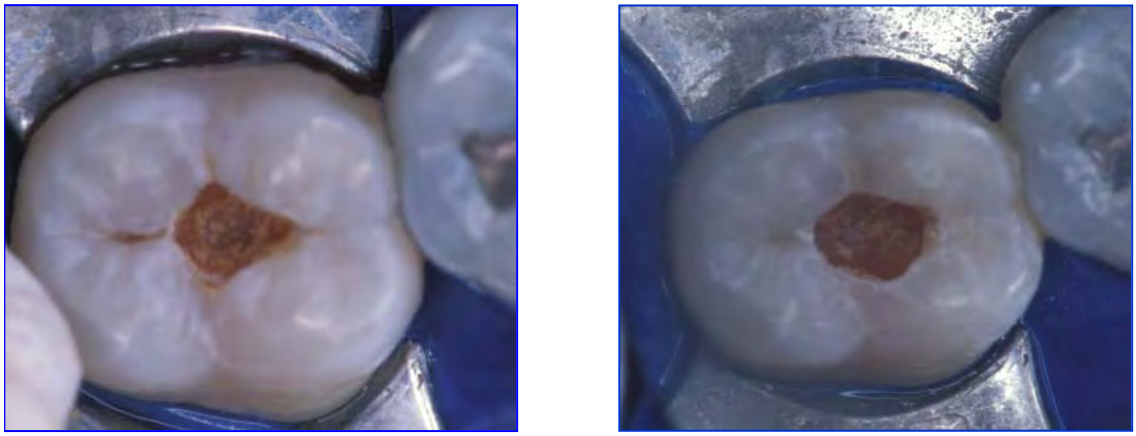

Figure 2.5 - Clinical photograph of tooth #31 at baseline (left) and re-entry (right)

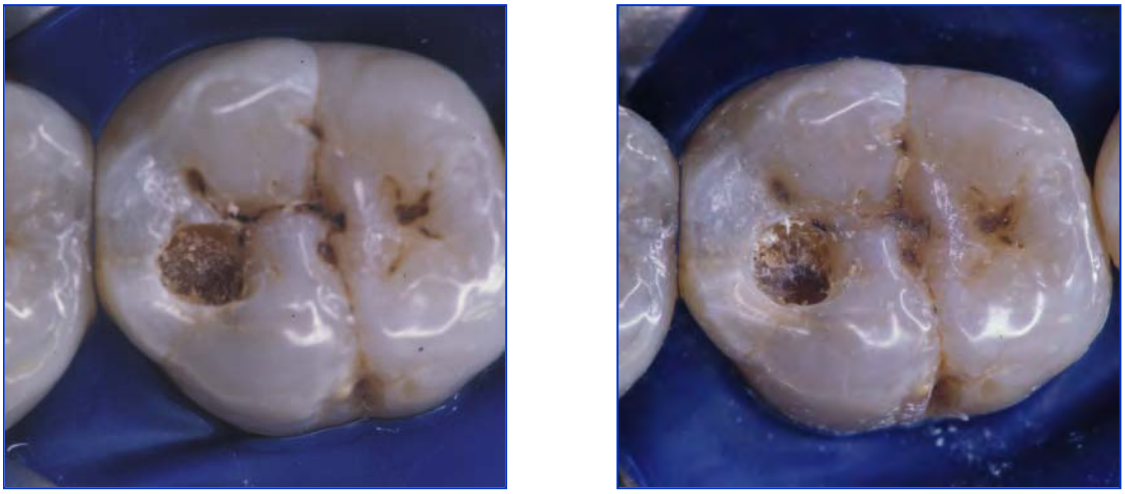

Figure 2.6 - Clinical photograph of tooth #30 at baseline (left) and re-entry (right)

No further instrumentation for caries removal or cavity prep was performed below the 1.7mm depth of the # 330 bur. The lesion activity, consistency, moisture and dentin color were evaluated by using written criteria **(Appendix G)**. Clinical evaluations of the carious lesions were made independently by two trained evaluators: the primary investigator (tl) and primary mentor (mp) and in case of disagreement, consensus assessment was obtained at the chair side.

2.3.2.3 Dentin Sampling

Prior to start of the study, the operator was trained in the biopsy technique on a series of freshly extracted human teeth with carious dentin lesions. For the study, two carious dentin samples were collected from each lesion for microbiological analyses. Fresh, sterile, round carbide #4 burs were used for collection of each carious dentin sample. As described by Kidd *et al.*, a bur was used in a slow speed (400 rpm) gear reduction hand piece to sample the carious dentin close to the central area of the lesion (Figure 2.5, Figure 2.6). A new bur was then carefully placed into the hand piece by using sterile thumb dressing forceps and dampened in phosphate buffered saline (PBS) solution. The bur was then pushed into the infected dentin and the adherent dentin constituted the sample. The bur was carefully removed from the hand piece by holding it with the forceps and was placed into 1.5 ml micro-centrifuge tubes (Eppendorf Safe-Lock) containing 500 µl of reduced transport fluid (RTF) (Figure 2.7). The same sampling procedure was repeated for the second dentin sample close to the central area of the lesion, not overlapping the first biopsy area. The samples were stored at -20^{0}C until the microbiological analysis. The locations of the samples were marked on the CRF/CEF form. A photograph of the lesion was taken following the dentin sampling.

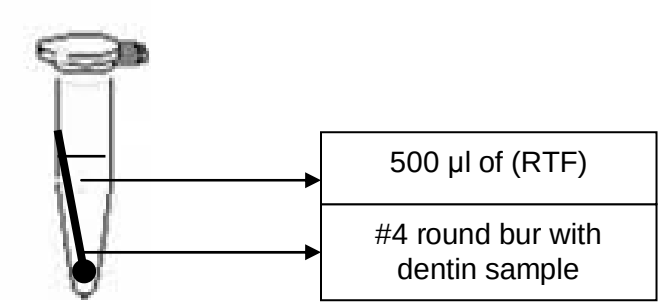

Figure 2.7 - Micro-centrifuge tube with RTF and dentin sample on the bur

2.3.2.4 Restorative treatment

A 37% phosphoric etchant liquid-gel (Scotchbond Etchant ™, 3M ESPE St. Paul, MN) was applied on the enamel for 15 seconds and the cavity was rinsed with air-water spray for 15 seconds. The cavity was blot dried and the adhesive (Single Bond Dental Adhesive ™, 3M ESPE St. Paul, MN) was applied as two consecutive coats to fully saturate both dentin and enamel. The cavity was gently dried with high speed suction for 2-5 seconds and light cured for 10 seconds. The cavity was restored with a nanocomposite (Filtek Supreme Plus ™, 3M ESPE St. Paul, MN) by placing 2-3 increments and each increment was light cured for 20 seconds. A darker than tooth color shade was selected for ease of removal of the restoration at re-entry. Following the necessary occlusal adjustments, finishing and polishing with an extra-fine diamond bur and the Enhance ™ finishing and polishing system (L.D. Caulk, Milford, DE), the margins of the restoration were re-sealed by re-etching and re-bonding with Scotchbond MP(3) and a photograph of the restoration was taken. After completing the restoration, an impression was taken using a triple tray with Imprint II (Garant Quick Step Impression Material System ™, heavy and light body, 3M ESPE, St. Paul, MN). Clinical assessment training using the Modified Ryge Criteria was performed on a number of clinical cases in student clinic patients with freshly placed and up to 6 month old restorations. Since such restorations usually do not show any sign of deterioration (especially not after re-bonding) a full calibration process of the evaluators was not deemed necessary. The operator and the primary mentor (mp) evaluated the restorations clinically based on the modified Ryge Criteria using the 0, 1, 2, 3 scoring system **(Appendix H)**. Patients were contacted by phone 1 week after the treatment and were interviewed for sensitivity or pain on the study tooth.

2.3.2.5 Clinical Procedure – Re-entry

After approximately 6 months, 16 out of 19 lesions were re-entered. Three teeth were lost to follow up: One patient with two class I lesions could not be reached for recall and one class I lesion was restored by an outside dentist. Following the medical and dental history update, patients were interviewed regarding the sensitivity of the study tooth for the last 6 months. Clinical evaluation of the restoration was made by the operator and the principal mentor (mp) based on the Ryge criteria **(Appendix H)**. Bitewing x-rays were taken by using the customized Rinn x-ray holder (previously prepared at baseline). The x-rays from baseline and re-entry were compared at the view box by the operator and the evaluator. The changes were recorded.

Electrical pulp testing was recorded for both study and contralateral teeth. Before removal of the restoration a photograph and an impression of the restoration were taken by using the same method as used at the baseline appointment. Local anesthesia was performed as needed. The restored teeth were re-entered under rubber dam isolation to prevent contamination with the saliva. A sterile # 330 bur was used with high speed to remove some composite material at the periphery of the restoration, creating a trough between the filling and the cavity wall. The restoration was then carefully lifted off the dentin with a sterile excavator or explorer placed in the prepared trough between the cavity wall and the restoration. The carious lesion was evaluated by using the same written criteria as used at the baseline treatment **(Appendix G)**. Two replicate re-entry samples of dentin were taken and stored using the biopsy procedures as described previously.

Following an intra-operative photograph of the remaining dentin, demineralized dentin was removed and the tooth was restored with a resin composite material following standard practice guidelines.

2.3.3 Microbiological Analysis

After the clinical procedures, the replicate dentin samples of each lesion were dispersed by vortexing for 1 minute. Following the removal of the bur (Figure 2.8), the samples were then combined in a single 1.5 ml micro-centrifuge tube and stored at -20^{0}C. Both the baseline and the re-entry samples were processed simultaneously. This eliminated potential sample bias in addition to avoiding minimal processing variations.

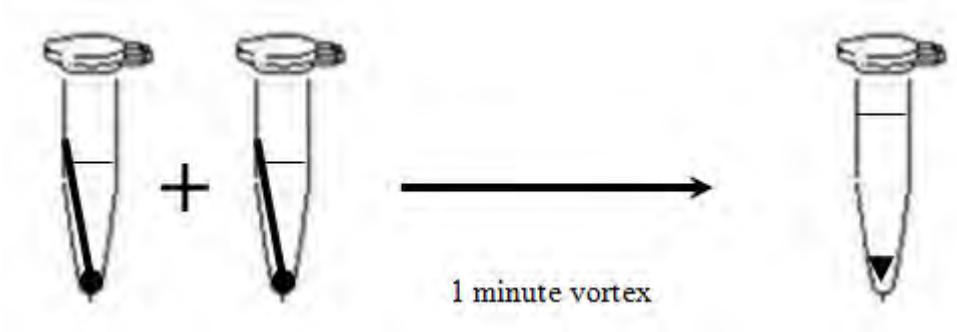

Figure 2.8 - #4 round bur was removed following the vortexing

2.3.3.1 Bacterial Lysis

After thawing, the cells in the dentin samples were harvested in a microcentrifuge tube by centrifuging samples at maximum speed (13,000 rpm) for 10 minutes, and the supernatant was discarded. The cell pellet was resuspended in 136 ul Buffer A. Buffer A was prepared with 20mM Tris (pH 8.0), 2mM EDTA, and 1.2% Triton X-100 in RNase free water (DEPC treated water), filtered sterile, and stored at 4°C. Streptococcal cells were quite resistant to standard lysis technique. Therefore the standard lysis buffer

supplied by the manufacturer (20mM Tris (pH8.0), 2mM EDTA, 1.25 Triton X-100, 25 mg/ml lysozyme) was supplemented with *mutanolysin* (Sigma - Chemical Co., St. Louis, 2500 U/ml) and *lyphostaphin* (Sigma - Chemical Co., St. Louis, 50 U/ml) to maximize DNA isolation. The samples were sonicated in a Branson model at 50% for three 10 second intervals following cleaning the tip of the sonicator with 70% ethanol and RNase away. 264 ul Buffer B was then added to the tubes. Buffer B was prepared with 200 ul 0.1% MDPB (methacryloyloxydodecyl pyridinium bromide) and 20 ul lysozyme, 20 ul mutanolysin, 20 ul lysotaphin and 4 ul ACP (Achromopeptidase). The samples were incubated at 37^0C for 30 minutes and centrifuged at maximum speed (13000 rpm) for 15 minutes at room temperature. The lysate was split into two portions (Figure 2.9):

1. ¾ of the lysate (300 ul) saved for RNA isolation at -70^0C
2. ¼ of the lysate (100 ul) saved for DNA isolation at -20^0C

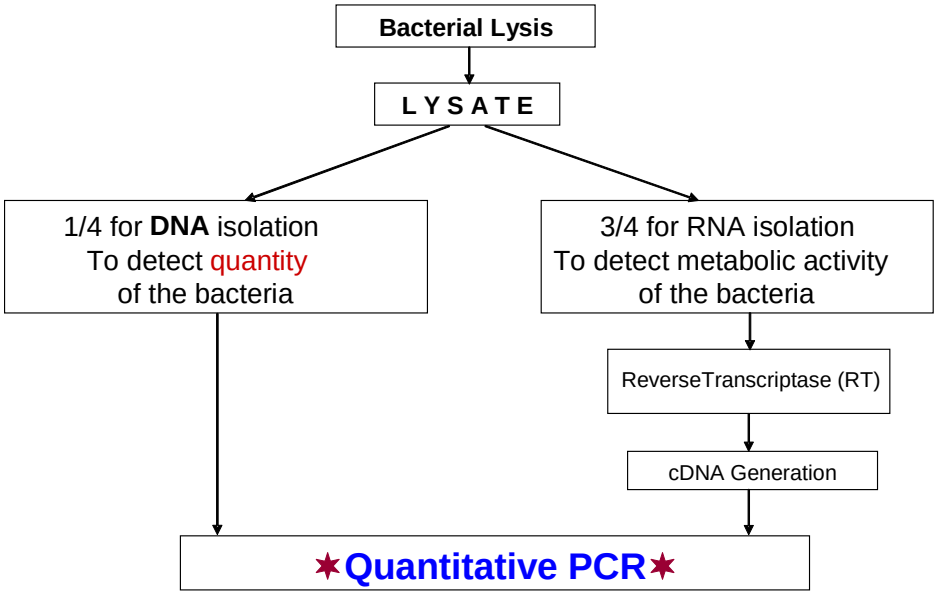

Figure 2.9 – Bacterial Lysis

2.3.3.2 RNA Isolation and cDNA Generation

Because of the chemical instability of RNA, special care was taken during the RNA isolation procedure. All bench surfaces and pipettes were treated with commercially available RNAse inactivating agents (RNAse Away, Molecular bioproducts, #7000). RNAse-free plasticware and reagents including tips and water were used at all times. Glassware was baked at 180-200^{0}C for 4 hours since autoclaving was not sufficient to inactivate RNAses. Prolonged exposure of RNA to high temperatures (>65^{0}C) was avoided since it may have affected RNA integrity. The RNA samples were always kept on ice while preparing the experiments.

150 ul DNase and RNase-free water and 250 ul ethanol (96-100) were added to the 300 ul lysate by mixing thoroughly. The samples were then applied to RNeasy mini column placed in 2 ml collection tubes (RNeasy RNA isolation kit by QAIGEN) and centrifuged for 15 seconds 8,000g (>10,000rpm). The flow-through was discarded and the collection tube was saved. 700 ul Buffer RW1 (RNeasy kit) was added into the column and centrifuged for 15 seconds at 8,000g (>10,000rpm). The column was then transferred into a new collection tube and added 500 ul Buffer RPE (RNeasy kit) and centrifuged for 15 seconds at 8,000g (>10,000rpm) to wash the column. The flow-through was discarded. The column was transferred again into a new collection tube and added 500 ul Buffer RPE one more time and centrifuged for 2 minutes at 8,000g (>10,000rpm) to dry the silica gel membrane. The column was transferred into a new 1.5 ml collection tube (RNeasy kit) and 30 ul RNase-free water was directly added onto the silica-gel membrane and centrifuged for 1 minute at 8,000g (>10,000rpm) to elute. If the isolated RNA was not processed right away, it was stored at -70^{0}C. Following the RNA isolation cDNA was generated.

8 ul isolated RNA, 1 ul 10X DNase I buffer (200mM Tris-HCl, 500mM KCl, 20mM MgCl2), and 1µl DNAse I (Amplification Grade, 1unit/µl) were combined together in a microcentrifuge tube and incubated for 15 minutes at room temperature. 1 µl of 25 mM EDTA was added for inactivation and heated for 10 min at 65^0C.

1 µl Random hexamers, 1 µl dNTP mix (10 mM each), 5 µl RNase free water and 5 µl DNase treated RNA from the previous step were combined together in 0.2 ml PCR tubes. The mix was then heated for 5 minutes at 65^0C and chilled on ice for 10 minutes. 4 µl 5x Strand Buffer, 2 µl 0.1 M DTT and 1 µl RNaseOUT were added to the PCR tubes and incubated at 25^0C for 2 minutes. 1 µl Superscript II RT was then added and incubated at the iCYCLER PCR machine; at 25^0C for 10 minutes, at 42^0C for 50 minutes, and at 70^0C for 15 minutes.

The generated cDNA was either processed immediately for real time PCR or stored at -70^0C.

2.3.3.3 DNA Isolation

DNeasy DNA extraction kit from Qiagen was used for DNA isolation. Before using the new kit, 96-100% ethanol was added to buffer AW1 and AW2. 100 ul lysate from bacterial lysis was mixed with 180 ul TE buffer by pipetting. 25 ul Proteinase K and 200 ul Buffer AL were added by vortexing and incubated at 70^0C for 30 minutes. 200 ul ethanol (96-100%) was then added and mixed by vortexing. The mixture was transferred into a DNeasy spin column placed in a 2 ml collection tube and centrifuge at 6000 g (8000 rpm) for 1 minute and flow-through was discarded. In a new 2 ml collection tube, 500 µl Buffer AW1 was added and centrifuged at 6000 g (8000 rpm) for 1 minute and the flow-through was discarded. In a new 2 ml collection tube 500 µl

Buffer AW2 was added and centrifuged at 13000 rpm for 3 minutes and flow-through was discarded. A spin column was placed in a 1.5 ml microfuge tube and 100 µl Buffer AE was added. The mixture was then incubated at room temperature for 1 minute and centrifuged for 1 min at 8000 rpm to elute. The extracted DNA was saved at -20^{0}C for further microbiological analysis.

2.3.3.4 Quantitative PCR

In order to detect the *quantity* of the bacteria in the clinical samples at baseline and re-entry, the isolated DNA samples were processed by using quantitative PCR. To detect the *metabolic activity* of the bacteria at baseline and re-entry isolated cDNA samples were processed by using quantitative RT-PCR. For each PCR run, a set of standard samples of DNA extracted from known number of bacteria were used to determine the concentration for the experimental samples. This method is called the *standard curve* method. In the present study, *S. mutans* is used to generate the standard curve. It is appropriate for use in quantifying *S. mutans* in quantifying total bacteria which include hundreds of species (we do not have a culture model for total bacteria).

To develop a set of standard samples; *Streptoccocus mutans* was grown from a single colony under aneorobic conditions at 37^{0}C. Following the plating and counting the number of colonies (CFU/ml), the concentration of original bacterial solution was calculated. DNA was isolated as described above and the quantitative PCR was processed to obtain the Cycle Threshold (Ct) value. A standard curve was generated on the Ct value corresponding bacteria concentrations (Figure 2.10).

For each PCR run, one negative control (1 µl RNase free distilled water instead of template) was used to check for possible contamination. All of the PCR reactions were carried out in triplicates. Two primer sets were used for both DNA and cDNA templates

Primer set I: CX 355 and CX 356 (*S. mutans* specific 16S rDNA primers)

Forward primer (CX355): 5'-AGAAAGGGACGGCTAACTA-3'

Reverse primer (CX356): 5'-CTTCTGCACTCAAGTCAGA-3'

Primer set II: CX 440 and CX 441 (universal 16S rDNA primers)

Forward primer (CX 440): 5'-TTGGGTTAAGTCCCGC-3'

Reverse primer (CX441): 5'-ATCCCCACCTTCCTCC-3'

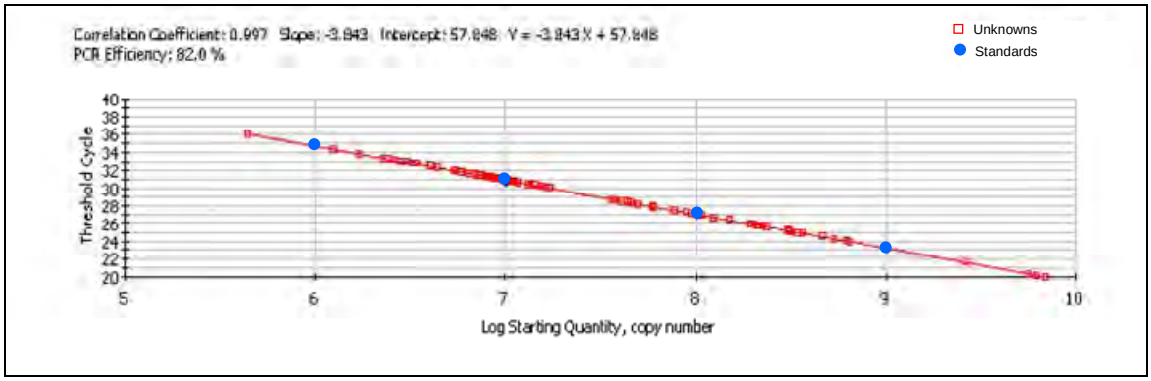

Figure 2.10 – Standard Curve Data

For the quantitative PCR reaction "QuantiTect SYBR Green PCR Master Mix" kit was used (Qiagen). For each DNA or cDNA sample with either universal and *S. mutans* specific primer set:

- 12.5µl 2x Sybr Green Master Mix
- 1 µl Forward primer
- 1 µl Reverse primer

As a master mix 9.5 µl sterilized RNase free water were prepared. Master-mix was kept on ice at all times. PCR plates were also loaded on ice. 24 µl master mix was added into each well. 1 µl template (DNA or cDNA) was added and mixed by pipetting. The triplicates were placed in the adjacent wells. iCycler PCR machine (Bio-rad) was used with the pre-set protocol for the quantitative PCR reaction.

Protocol for the PCR reaction:

Cycle 1: (1X) Step 1: 95.0ºC for 15:00 minutes

Cycle 2: (40X) Step 1: 95.0ºC for 00:30 seconds, Step 2: 50.0ºC for 00:30 seconds, Step 3: 72.0ºC for 00:30 seconds

Cycle 3: (1X) Step 1: 72.0ºC for 08:00 minutes

Cycle 4: (1X) Step 1: 95.0ºC for 01:00 minute

Cycle 5: (70X) Step 1: 95.0ºC for 00:10 seconds

Cycle 6: (1X) Step 1: 4.0ºC HOLD

Following this protocol, the amplification graph below (Figure 2.11) was obtained.

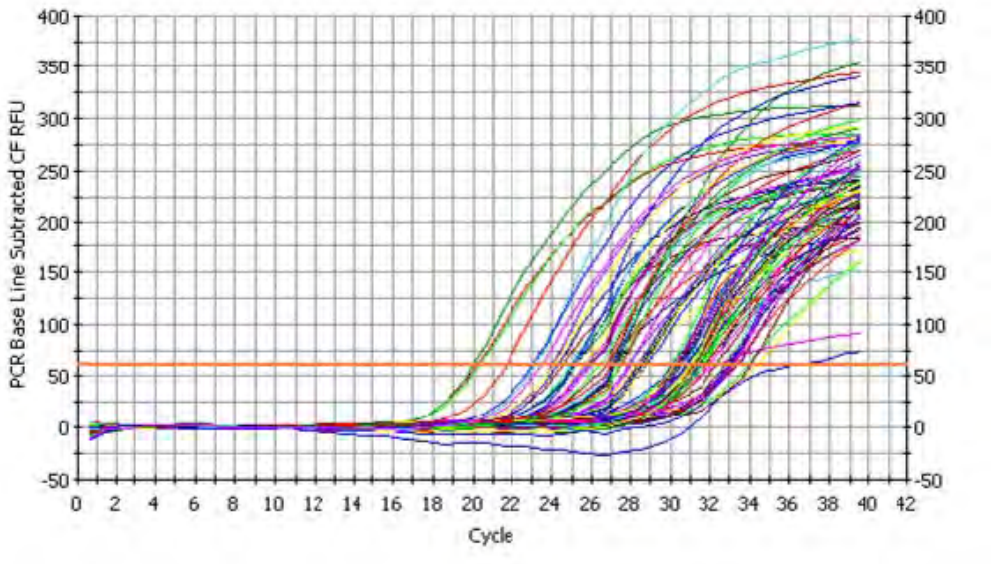

Figure 2.11 - End result: Quantitative RT PCR amplification cycle graph

2.3.4 Data Analysis

The outcomes for the present study are bacterial presence and activity in infected dentin, clinical characteristics of carious dentin, and radiographical data of lesion change.

For the sample size calculation we originally referred to culturing data from Bjørndal *et al.,* 2000. Sample size calculations were based on the paired t-test of the logarithms of the observations. A conservative estimate for standard deviation (SD) = 1.5. This provides an effect size of ES = 0.67. Then for alpha = 5%, and a two-sided test, group size n = 24 would give 90% power to detect a difference in logs of one unit using a two-sided paired t-test on the logs. Thirty teeth with occlusal cavities were originally planned to be included at baseline. If we assume 20% loss after six months, the re-entry recall will include 24 teeth. Thus, an intake of 30 teeth was planned to provide a full set of outcome data for 24 teeth.

For the microbiological evaluation of the dentin biopsies the repeated measurement procedures in SAS (version 9.02) were performed to compare the differences in total bacterial load at baseline and at re-entry after six months. Data were collected of the total bacterial load and bacterial activity. In the present study the bacterial load in dentin was expected to be reduced after 6 months.

The clinical evaluations include tooth sensitivity, descriptors for carious dentin, and restoration quality scores. Descriptive statistics were used to compare baseline with recall evaluations.

Radiographic evaluation of the lesions by standardized bite-wing radiographs (BWs) was used to monitor lesion change. The radiographs were used to diagnose the

caries lesion and assess its dimensions at baseline, and subsequently to monitor potential change in dimension and density of the dentin.

2.4 Results

2.4.1 Clinical Results

2.4.1.1 Tooth Response

A total of sixteen lesions were evaluated for the results of the study.

Interview – Based on the interviews with patients regarding tooth sensitivity at the 6 month re-entry visit, none of the lesions showed development of clinical symptoms such as sensitivity to cold and hot. Patients were asked to describe sensitivity level on a 0-10 scale (0: no sensitivity, 10: extreme sensitivity) during baseline and re-entry visits. Four of the 16 lesions were given a score of 1 and the rest of the lesions were given a score of 0 to sensitivity at baseline. At re-entry all of the lesions were given a score of 0 to sensitivity to hot and cold.

Electrical pulp testing (EPT) responses – Electrical pulp testing readings were within the normal clinical range at both baseline and re-entry visits (Table 2). Only three out of sixteen lesions showed a minimal increase response time. None of the lesions developed pulpal necrosis after sealing the caries for 6 months.

2.4.1.2 Restoration Performance

The application of adhesive restorative system to irreversibly infected dentin did not affect the clinical performance of the restoration during the 6 month period. All restorations were evaluated at baseline and re-entry using 4 different criteria: margin discoloration, margin integrity, caries and anatomic form; all restorations received score "0" at re-call **(Appendix H)**.

Table 2 - Electrical pulp testing results

Lesion	Baseline	Re-entry	Contra lateral tooth	Baseline	Re-entry
1	24	33	**1**	28	8
2	25	34	**2**	13	41
3	33	29	**3**	38	26
4	53	54	**4**	80	44
5	33	42	**5**	67	48
6	55	33	**6**	47	33
7	38	46	**7**	69	44
8	49	46	**8**	62	69
9	50	41	**9**	13	41
10	39	46	**10**	31	36
11	33	48	**11**	70	25
12	67	40	**12**	55	40
13	40	43	**13**	46	46
14	21	41	**14**	36	33
15	10	32	**15**	30	38
16	24	33	**16**	17	28

2.4.1.3 Radiographic Results

None of the 16 lesions developed radiographically detectable lesion progression.

2.4.1.4 Lesion Change

At baseline, 75% of the lesions were soft while only 12.5% were evaluated as soft at re-entry. Twelve point five percent of the lesions were hard at baseline a number that increased to 56% at re-entry. Lesions that showed medium consistency increased from 12.5% at baseline to 31% at re-entry (Figure 2.12).

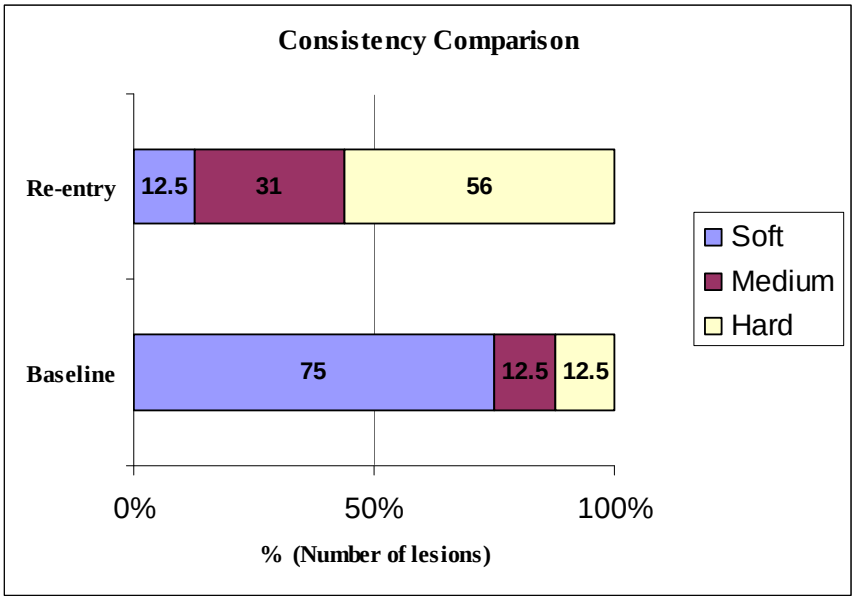

Figure 2.12 - Consistency comparison data at baseline and re-entry

Lesion moisture content dropped from 43% wet at baseline to 12.5% wet at re-entry while the number of dry lesions at baseline increased from 56% to 87.5% at re-entry.

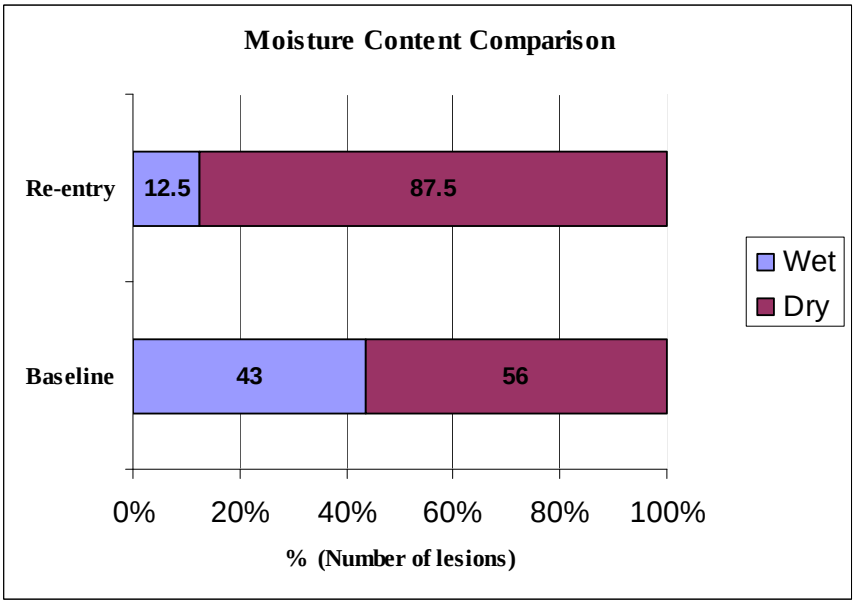

Figure 2.13 - Moisture content comparison data at baseline and re-entry

The color shift showed a lighter trend at re-entry compared to baseline. 62% of the lesions were dark brown at baseline and 56% of the lesions show a light brown color at re-entry (Figure 2.14).

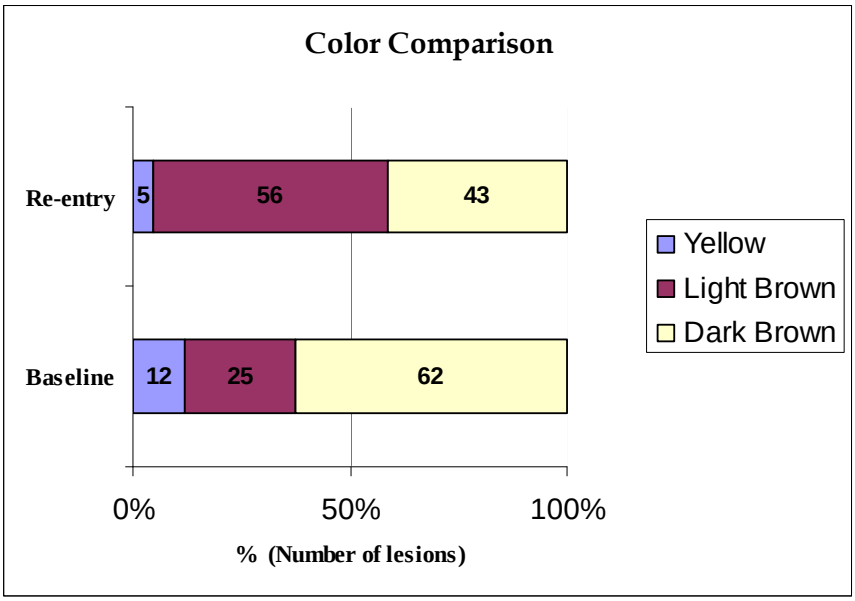

Figure 2.14 - Color comparison data at baseline and re-entry

2.4.2 Microbiological Results

2.4.2.1 Total Bacteria

The differences between the amounts of DNA and RNA of *total bacteria* at baseline and re-entry were analyzed using Repeated Measurement Procedures in SAS (version 9.02).

S. mutans and total bacteria proportion, for DNA and RNA at baseline and re-entry were compared by using paired t-test analysis. Statistical significance was determined at the $p \leq 0.05$ level.

There was no statistical difference found on the total bacteria at baseline and re-entry for both DNA (P=0.3714, power=0.16) and RNA (P=0.1551, power 0.4) (Figure 2.15).

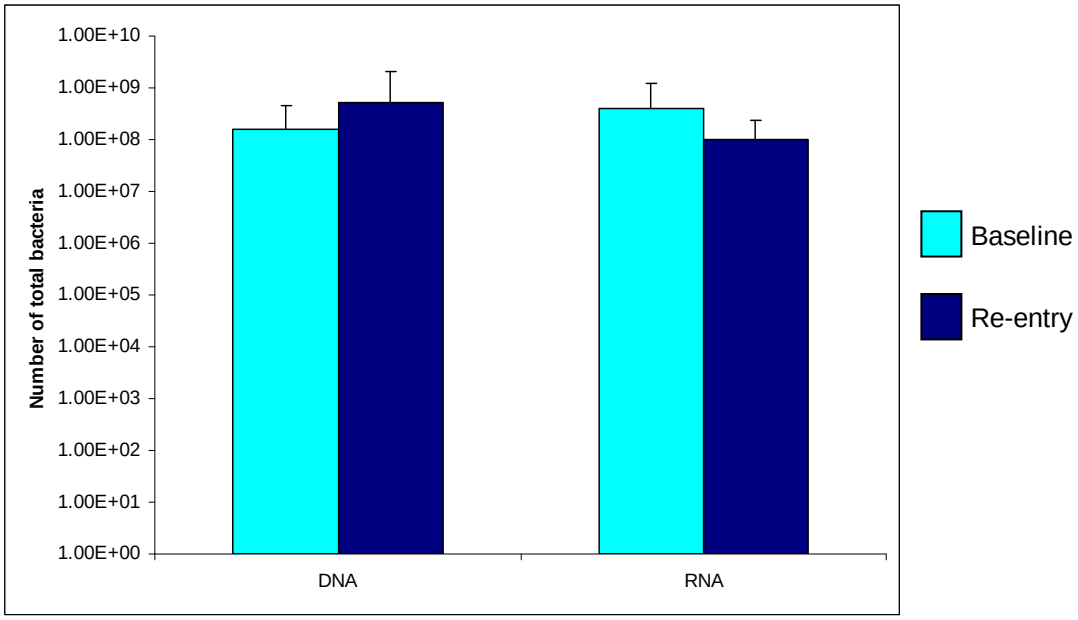

Figure 2.15 - Comparison of total bacteria at baseline and re-entry

2.4.2.2 Streptococcus mutans

The observed decrease in S. *mutans* proportion of total bacteria at re-entry compared to baseline was not statistically significant (P (T<=t) =0.1158, power is 0.4). (Figure 2.16, DNA).

For the metabolic activity of the *S. mutans* (RNA), there was statistically significant (P=0.0165) decrease at re-entry compared to baseline. Power is 0.7 (Figure 2.16, RNA).

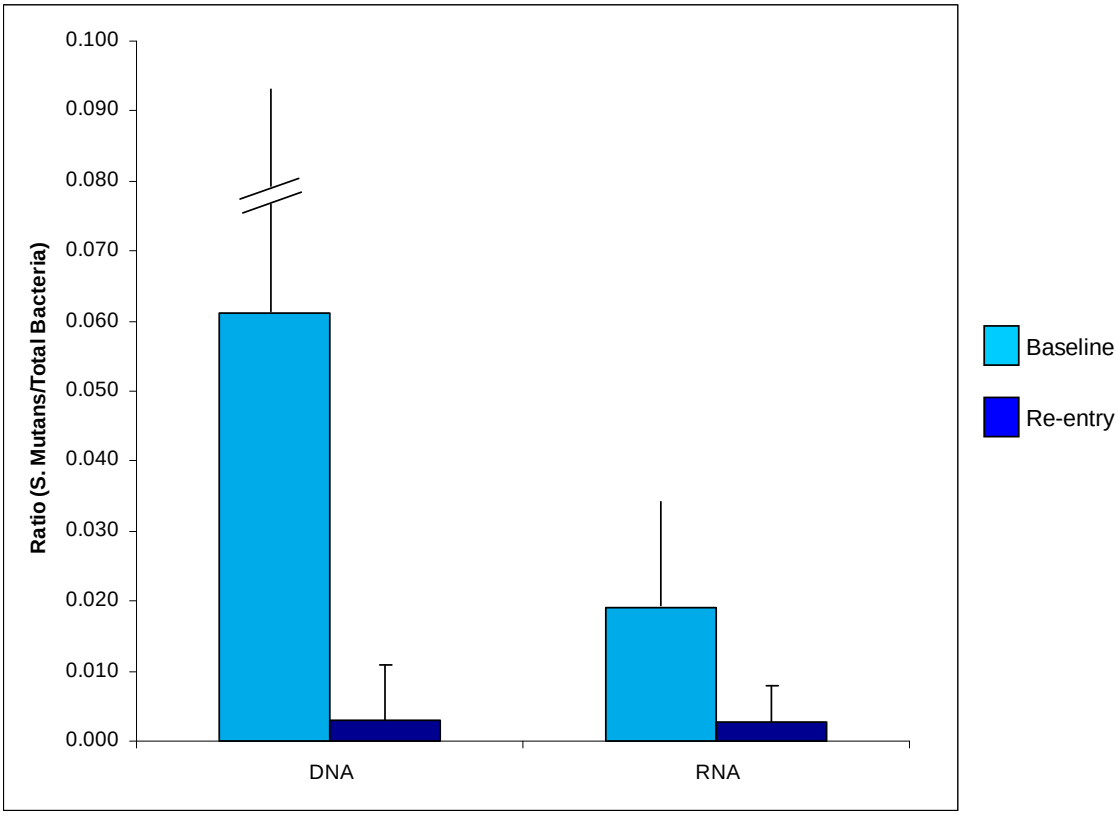

Figure 2.16 - *S. mutans* / Total bacteria proportion – for baseline and re-entry.

Standard deviations: DNA-BL: 0.139, RE: 0.008, RNA-BL: 0.024, RE: 0.00

2.5 Discussion

2.5.1 Clinical Treatment

Numerous studies on sealing carious dentin demonstrate quantitative reduction of total bacteria and *S. mutans*, change of dentin tissue texture to a harder consistency and no progression of the lesion after a good peripheral seal [3, 7, 8, 15-21]. Sealing off caries and associated bacteria in a cavity with an ultraconservative restoration that provides sealing, arrests lesion progression over a period of 10 years [7]. There is no clinical, micobiological, or radiographical evidence in the literature that partial caries removal accompanied by effective sealing is deleterious in terms of signs and symptoms of pulpitis or necrosis [22].

In the present study, the primary goal was to compare microbiological and clinical changes in carious lesions before and 6 months after ultraconservative tooth sealing. Using molecular biology techniques, the quantity and metabolic activity of bacteria in affected dentin was assessed at baseline and 6 months after the cavity was sealed. Based on quantitative PCR results, the sealed bacteria survived over the 6 month time period. We observed that *S. mutans* survived under the sealed restorations, but the metabolic activity of *S. mutans* was significantly reduced compared with baseline. This finding was in accordance with hard and dry clinical appearance of the carious dentin at the 6 month re-entry.

This study included 19 occlusal lesions at baseline, which came from 9 patients. After 6 months, two patients (3 lesions) could not be contacted; therefore only 16 lesions were evaluated and included in the final data analysis. Clinical studies that include recalls are prone to loss of data due to the inherent problem of patients often not

presenting for recall and thus becoming “lost-to-follow-up”. The present clinical design accounted for estimated patient drop-out when the power of the study and its sample size were determined. The original design of the study, however, was based on comparison of tooth lesions at baseline and 6 months after treatment using a culture-based study. Although this data was not comparable to PCR-outcomes, it was the only data available at that time to use as a basis for sample size calculations. Keeping this approach in mind led to the decision to finish the group of study lesions at a total of 19 at baseline, rather than the 30 teeth originally planned. Therefore, the results of this study may be considered preliminary, as the original sample size was not able to be obtained for the analysis.

The carious lesions in this study were not confined to the occlusal surface. In addition to 13 occlusal surface lesions requiring an access opening to be made, 3 cervical lesions were included which did not require an access opening. The occlusal and cervical lesions in this study may have experienced a different bacterial environment due to their size and location in the oral cavity, and their association with the dental plaque. However, no quantifiable difference in bacterial numbers was detected between occlusal and cervical lesions based on the quantitative PCR results. Although a few of occlusal lesions were relatively small compared to the rest of the study lesions, quantitative analysis of the total bacterial load of each lesion revealed a high degree of bacterial presence, and thus infection, at baseline.

A recent systematic review pointed out that few randomized controlled clinical trials (RCT) concerning sealed caries were reported in the literature [22]. The authors found a total of 4 RCTs that fit their criteria, but the method of randomization in these selected

studies was not standard. The size of the lesions included varied as some lesions were deep enough to be judged clinically to result in pulp exposure at caries removal, whereas other lesions extended only halfway into dentin. The restorative materials used in the studies also varied. Some studies used glass ionomer material to seal the caries while the comparison studies used amalgam or composite for the same purpose. There were insufficient data to make conclusions about the respective benefits of each material.

Other systematic reviews also revealed that the amount of caries removed in incomplete caries removal varied [10]. In studies that used a step-wise excavation technique most of the soft dentin was removed. In contrast, in some other studies the dentin-enamel junction was made caries-free but soft dentin was left on the pulpal wall. In the Mertz-Fairhurst study caries removal was ultraconservative [7].

Ideally, the patient, the operator and the evaluator in a clinical trial should all be "blinded" as to any treatment intervention. However "total blinding" was not possible in the reported studies as the operator knew whether caries removal was partial or complete. Conventional blinding of the operator was precluded. Moreover, the operator was often also one of the two evaluators, as in the present study. None of the reported studies managed to avoid this bias entirely. In the Mertz-Fairhurst study, amalgam was used in control cavities and composite in the treatment group, and thus evaluator blinding was not possible [7]. In the present study, we could only have avoided the "time point" bias in the clinical results if baseline and re-entry patients would have been treated in the same time period. The logistics and extended length and sample size of such a study made this virtually impossible. The laboratory part of the study, however,

was performed with coded, not-identifiable samples. Concurrent processing of the baseline and re-entry samples minimized potential sample bias.

Sampling of carious dentin for microbiological assessment was first described in detail by Kidd *et al.,* 1993. It was shown by repeated laboratory tests (weighing of samples) that this method was sufficiently accurate to make valid comparisons between samples from soft and hard dentin [23]. In order to confirm the accuracy of this sampling method Bjørndal *et al.,* 1997, weighed dentin samples collected as described by Kidd and demonstrated that similar amounts of material could be obtained from sampling sites with different consistency [3]. This is the only validated sampling method in the literature.

In the present study a standardized sampling procedure as described above was used to obtain duplicate dentin samples from each site. The reason for collecting duplicate samples from each lesion at baseline and re-entry was to obtain a reasonable amount of bacteria from lesions and avoid high variability between samples. Except for few outlier data points, detected at both baseline and re-entry, it can be concluded that the dentin biopsy procedure in the present study achieved reliable results since, in general, we observed relatively consistent bacterial numbers. At baseline the location of the sampling site was marked on the clinical forms and at re-entry adjacent dentin was sampled. For smaller lesions, however, biopsy was taken close to the baseline location and may have resulted in sampling of slightly deeper dentin. However, despite these limitations, this method is currently the most reliable amongst similar methods found in the literature.

2.5.2 Molecular Microbiology

Recent systematic reviews included studies which were conducted before year 2000. The sole criterion for bacterial viability in these studies was cultivability. Since that time DNA-based microbiological quantification and characterization have been validated, eliminating the need for cultivation, which captures only the small minority of cultivable microorganisms present. In the present study, molecular biology techniques were used to detect quantity and metabolic activity of bacteria at baseline and re-entry intervals.

Among the diverse oral microbial community, streptococcal cells are known to be quite resistant to standard lysis techniques. In order to obtain greater sensitivity different lysis methods have been reported in the literature. Mutanolysin was tested by Yokogawa *et al*., 1974, against cell suspensions of various living microorganisms to obtain its lytic spectrum [24]. All gram-negative organisms were resistant to lysis. Mutanolysin had lytic or bactericidal activity against living cells of *S. mutans*. Most of the recent studies concerning *S. mutans* modified the standard lysis technique by including mutanolysin to maximize lysis and thus DNA yield. Some studies, however, used the "Bead Beater" lysis technique which requires extensive vortexing with very small glass beads [25]. The present study used mutanolysin to maximize the bacterial lysis, because it is currently the best method available for oral bacterial lysis.

After introduction in biomedical research, PCR techniques have become mainstream research techniques and are frequently used in dental research in the last decade. In addition to a traditional PCR, quantitative PCR provides information beyond mere detection of DNA. It indicates not just whether a specific DNA segment is present in a sample, but also how much of it is there and can thus be used to quantitate all numbers.

In the present study, reliable means of quantifying bacterial DNA, and therefore bacterial numbers, at baseline and re-entry for total bacteria and *S. mutans* were employed using the quantitative PCR technique. Currently, this is the most accurate and reliable method to quantify the bacterial cell numbers.

Similar studies have assessed the quantity of bacteria after cavity seal by using molecular biology techniques. However, none of these studies assessed the relative metabolic activity of the bacteria and their ability to cause disease progression. Assessment of metabolic activity of bacteria using Reverse-Transcriptase PCR (RT-PCR) is relatively difficult due to the extreme sensitivity of RNA to endogenous nucleases. Optimal conditions are needed to avoid breakdown of RNA and to remove DNA contamination from the RNA samples. In addition, it is more difficult to extract nucleic acids from dental tissue than from plaque or soft tissue samples. The unique nature of the present study is that RT-PCR technique was carried out on the RNA samples and that the metabolic activity of total bacteria and *S. mutans* was assessed for baseline and re-entry samples. This is novel, cutting edge work, though more development is needed.

One of the clinical findings of the current study is that carious dentin appeared harder and drier after 6 months of being sealed (Figure 2.12). This outcome was consistent with similar studies in the literature. In contrast to the results of previous studies, we found that the color of dentin appeared lighter at re-entry (Figure 2.14). This finding corroborates the statement that "color of carious dentin does not relate to the level of infection" [23], nor to caries activity of root lesions [26].

In general, culture-based studies reported reduction (or even elimination) of total bacteria after sealing off the cavity [2, 3, 19, 21, 27]. In contrast to these studies, we observed that the quantity of 'total bacteria' showed no difference at re-entry (Figure 2.15, p=0.3714). Due to limited sample size, this study may be considered to be preliminary. Although no significant results were achieved for total bacteria, some results (RNA) seemed encouraging and in the expected direction. Future studies with a larger sample size might provide more definitive answers to questions related to microbial changes in total bacterial DNA and RNA 6 months after sealing. In addition it is important to remember that culturing techniques only allow the count of viable bacteria, while PCR techniques identify both viable and nonviable bacterial cells. Using regular PCR techniques only, it is difficult to state what percentage of these numbers at re-entry indicates dead bacterial cells. Therefore, assessment of metabolic activity of the bacteria at re-entry becomes a key to our understanding of the mechanisms involved.

Even though the quantity of the *S. mutans* at re-entry were reduced, the difference was not significant due to high degree of inter-sample variation and low sample size. The major finding of the current study based on the quantitative RT-PCR results was that the major cariogenic bacteria, *S.mutans,* was metabolically less active after 6 months under a sealed restoration (Figure 6, p=0.0165). This finding is very important since this study is the first to evaluate the metabolic (and presumable cariogenic) ability of bacteria entombed under a restoration. This result supports the hypothesis of the study that sealing provides a way to arrest metabolic activity of bacteria in caries lesions and to stop caries progression.

The effect of drastic environmental change on bacteria under a sealed restoration is not yet well-understood. The effect of sudden cut-off of nutrition supply on cariogenic ability of the metabolically active bacteria has been a subject of numerous research projects. The present study showed significantly reduced metabolic activity of *S. mutans* after 6 months of starvation. This raises questions regarding the time-effect relationship. Did an exponential reduction occur immediately after sealing or did the reduction happen at a linear rate over the 6 month period? In order to better understand the reduction pattern, additional RCTs with different re-entry intervals (*e.g.* 1, 3, 6, 9, 12, 18, 24 months) and a larger sample size are warranted.

The findings of the present study are important since we showed that it is possible to assess the metabolic activity of bacteria in dentin samples. Even though the technique employed is very sensitive and needs to be enhanced, this preliminary study provides a necessary basis for future studies. Our goal for this study was to use the currently available techniques to investigate the most studied cariogenic bacteria, *S. mutans*, and 'total bacteria'. Assays of quantitative RT-PCR of genes encoding specific enzymes of *S. mutans* and other pathogenic bacteria of caries also might be included in future studies in order to more clearly focus on expression of virulence factors.

Upon cavity sealing, the bacteria are exposed to a significant environmental change and severe nutrient limitation. The available nutrients, or the relative simplicity and homogeneity of nutrient supply significantly affect surviving bacteria [28]. The fate of the "entombed" bacteria after peripheral seal and their survival without nutrients from the oral environment are topics that require further research.

In summary, 6 months after ultraconservative cavity sealing, some entombed bacteria managed to survive, but they were relatively metabolically inactive. It can be concluded that sealing of carious dentin arrests lesion progression, thus providing a positive local environment allowing affected tissue to heal and remineralize.

2.6 Conclusions

Within the limitations of this study, the following conclusions can be made:

1. The use of quantitative PCR on both exposed and sealed dentin detected the number of total bacteria as well as the metabolic activity of the bacteria at baseline and at re-entry for the first time. The unique finding of this study is demonstration of the ability to evaluate the likelihood of cariogenic potential of bacteria by using molecular biology techniques.

2. A significant decrease of *S. mutans* metabolic activity (RNA) over a 6 month period indicated that major cariogenic bacteria were not completely eliminated from the cavity but showed a decreased cariogenic potential. The bacteria are not likely to cause any further demineralization under the sealed restoration.

3. After 6 months under sealed resin restorations, the carious dentin was harder and drier, which suggests healing and possibly remineralization.

4. Transitional composite restorations placed over soft carious dentin were clinically acceptable after 6 months.

2.7 Clinical Significance

The combination of both clinical changes in demineralized dentin and reduced metabolic activity of 6 month entombed cariogenic bacteria indicate arrest of the caries process.

2.8 References

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2.9 Appendices

Appendix A: Informed Consent Form

Appendix B: Incentive Form

Appendix C: Clinical Protocol

Appendix D: Clinical Sequence Form

Appendix E: CRF, CEF Baseline

Appendix F: CRF, CEF Re-entry

Appendix G: Lesion Evaluation Form

Appendix H: Restoration Evaluation Form