EVALUATION OF SALIVARY FLORA ACIDOGENICITY UNDER ACIDIC CONDITIONS FOR PREDICTION OF CARIOGENIC POTENTIAL DURING FIXED ORTHODONTIC TREATMENT

Submitted in partial fulfilment to the degree of Doctor of Clinical Dentistry (Orthodontics)

By

Sara A. Roberts
B.D.S.

School of Dentistry
Faculty of Health Sciences
The University of Adelaide
South Australia
5005

2010
# Table of Contents

Figures and Tables ........................................................................................................... 5  
SECTION 1 .......................................................................................................................... 5  
SECTION 2 .......................................................................................................................... 5  
Abstract ............................................................................................................................ 7  
Declaration ......................................................................................................................... 9  
Acknowledgements ........................................................................................................... 10  
SECTION 1 ....................................................................................................................... 11  
Orthodontics and caries risk ......................................................................................... 11  
  Introduction ....................................................................................................................... 11  
  Incidence .......................................................................................................................... 11  
  Brackets and bands .......................................................................................................... 13  
  Cements ............................................................................................................................ 14  
  Plaque and bacterial numbers ......................................................................................... 15  
  Distribution of teeth affected ......................................................................................... 16  
  Post orthodontic treatment analysis .............................................................................. 17  
  Multifactorial aetiology .................................................................................................... 18  
Oral flora associated with dental caries ........................................................................ 18  
  Diversity of micro-organisms involved in dental caries .................................................. 19  
  Historical and current nomenclature of mutans streptococci ....................................... 19  
  Mutans streptococci ........................................................................................................ 20  
  Lactobacilli ...................................................................................................................... 22  
  Other cariogenic bacteria ............................................................................................... 23  
Variation within individual microbial species ................................................................ 24  
  Mutans streptococci ........................................................................................................ 25  
  Lactobacilli ...................................................................................................................... 26  
Caries Prediction .............................................................................................................. 27  
  Prediction studies ........................................................................................................... 28  
  Predictors ....................................................................................................................... 28  
  Accuracy of caries prediction ......................................................................................... 29  
  Sensitivity and specificity in a clinical setting ................................................................. 30
Figures and Tables

SECTION 1

Figures
2. Typical follow-up for evaluating the predictive power of a dichotomous risk marker for caries (Hausen, 1997)

Tables
1. Sensitivity, Specificity, positive and negative predictive values for different types of studies with lactobacilli and/or mutans streptococci as caries-risk predictors (van Houte, 1993)

SECTION 2

ARTICLE 1

Tables
1. Initial pH change, risk category, and white spot lesion development in patients undergoing fixed orthodontic treatment.
2. Change in salivary pH for each subject over time.
ARTICLE 2

Tables

1. Results for high risk group, showing mutans streptococci CFU/ml, optical density of culture, terminal pH of broth, production of lactic and acetic acid from each strain of mutans streptococci.

2. Results for low risk group, showing mutans streptococci CFU/ml, optical density of culture, terminal pH of broth, production of lactic and acetic acid from each strain of mutans streptococci.
Abstract

Orthodontic treatment is a common occurrence with up to 29.7% of the adolescent population (Bollen, Cunha-Cruz et al., 2007) and 1% of the adult population (Whitesides, Pajewski et al., 2008) receiving fixed braces. This type of treatment poses significant risks to the hard and soft tissues. One of the most common complications of fixed orthodontic appliance treatment is the demineralization and subsequent white spot lesion development in the enamel (Travess, Roberts-Harry et al., 2004). White spot lesions are the early sign of dental caries and the incidence of white spot lesions in orthodontic patients has been reported as being as high as 50 per cent (Gorelick, Geiger et al., 1982; Lundström and Krasse, 1987; Lovrov, Hertrich et al., 2007) with white spot lesions sometimes occurring as early as 1 month after banding (Ogaard, Rølla et al., 1988).

Currently available chair-side saliva tests measure bacterial counts or acid production of the entire oral microflora. These tests tend to be able to predict patients who are at a low risk of demineralization more accurately than those at an increased risk. There is no single test to suit all individuals that can reliably identify at risk patients (Hausen, 1997; Reich, Lussi et al., 1999; Zimmer, Bizhang et al., 2008).

The aim of this short-term study was to evaluate a technique to predict white spot lesion development in patients undergoing fixed appliance orthodontic treatment, and to analyse salivary bacteria to determine any differences in their metabolism.

Fifty-two patients due to start fixed appliance orthodontic treatment agreed to participate in the study. Saliva samples collected before braces were placed
and during treatment at six-eight week intervals, were mixed with a potassium phosphate buffer solution containing sucrose (10% w/v), at pH 5.7, and rate of pH change was measured over 30 minutes. Demineralisation development was determined from standardized intra-oral photographs.

Subjects whose samples showed the greatest pH change towards acid production were selected for further salivary analysis. Ten of the higher risk individuals were further analysed, along with ten low risk individuals. Samples were grown on TSY20B plates, and pure strains of mutans streptococci were isolated and re-grown. These were then suspended in tryptone-soya broth and after 48 hours optical density, terminal pH and acid analysis using HPLC were measured and analysed.

Of the fifty-two participants, three developed demineralisation. Two were identified as high risk from their initial saliva test, one as low risk, giving the test a sensitivity of 67% and specificity of 94%. There was no statistically significant change over time in the subjects, which indicated the risk status is unlikely to change.

There was no statistically significant difference between the high and low risk groups in salivary microflora metabolism. The major acid produced in each case was lactic acid, with acetic acid being produced at lower concentrations.

This test has the potential to be developed into a commercial chair-side saliva test. However, further testing is continuing and aims to follow the cohort of patients through the entirety of their treatment.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Sara Roberts and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Dr Sara Roberts

Dated
Acknowledgements

I would like to thank the following people for their support during the past three years.

Professor W. J. Sampson, P.R. Begg Chair in Orthodontics, The University of Adelaide, for his time, advice and expert opinion.

Dr N.J. Gully, Senior Lecturer in Microbiology, The University of Adelaide, for his time, advice, laboratory assistance, guidance and expert opinion.

Associate Professor C. Dreyer, Senior Lecturer in Orthodontics, The University of Adelaide, for his advice and expert opinion.

Thomas Sullivan, Statistician, Data Management & Analysis Centre, Discipline of Public Health, University of Adelaide for statistical advice and analysis.

The ASO foundation for Research and Education, for financial support.

To my family for all their support and understanding over the past three years.
Orthodontics and caries risk

Introduction

Fixed orthodontic banding is a common occurrence with up to 29.7% of the adolescent population (Bollen, Cunha-Cruz et al., 2007) and 1% of the adult population (Whitesides, Pajewski et al., 2008) receiving treatment. It is predicted that the number of adolescents receiving orthodontic treatment will increase by approximately 0.7% per year (Bollen, Cunha-Cruz et al., 2007). This type of treatment poses significant risks to the hard and soft tissues. One of the most common complications of fixed orthodontic appliance treatment is the demineralization and subsequent white spot lesion development in the enamel (Travess, Roberts-Harry et al., 2004). White spot lesions are the early sign of dental caries. Their appearance is caused by an optical phenomenon due to subsurface mineral loss in the enamel (Gorelick, Geiger et al., 1982).

Incidence

The incidence of white spot lesions in orthodontic patients has been reported as being as high as 50 per cent (Gorelick, Geiger et al., 1982; Lundström and Krasse, 1987; Lovrov, Hertrich et al., 2007) and it has been reported that white spot lesions can occur early in treatment, sometimes as early as 1 month after banding (Ogaard, Rølla et al., 1988).
It is a common thought that the placement of a full fixed appliance makes the mechanical removal of plaque more difficult (Lara-Carrillo, 2010) and could, therefore, increase the caries risk. However, the increased focus on oral hygiene during the course of orthodontic treatment could in fact reduce the caries frequency. Several authors have reported that they found no increase in caries frequency in patients undergoing orthodontic treatment (Bach, 1953; Zachrisson and Zachrisson, 1971); however, many others have a differing opinion (Bach, 1954; Ingerval, 1962; Gorelick, Geiger et al., 1982; Lundström and Krasse, 1987; Ogaard, Rølla et al., 1988 (a); Ogaard, Rølla et al., 1988 (b); Travess, Roberts-Harry et al., 2004; Lovrov, Hertrich et al., 2007). The methods used in some of these previously mentioned studies have put into question the validity of the results. Bach (1954) routinely performed dental prophylaxis procedures on his patients and didn’t include cases with inadequate oral hygiene, while the Zachrisson and Zachrisson (1971) study provided both fluoride applications and hygiene instructions to the orthodontic patients but not the control group (Southard, Cohen et al., 1986).

Southard et al. conducted their study to look at the relationship between fixed appliance orthodontic treatment and caries incidence based on DMF indices, among a nationwide pool of naval recruits (Southard, Cohen et al., 1986). They found that the orthodontically treated patients had better dental health, with respect to caries development, than non-orthodontically treated patients. Orthodontic patients had fewer overall diseased surfaces than the control group, with a difference averaging one less carious surface per patient (Southard, Cohen et al., 1986). It was also noted that there were significantly fewer cases of occlusal caries, suggesting better preventive care and an
equally accessible site for preventive treatment in both groups. There was also a shift in the orthodontically treated group away from interproximal caries development to facial-lingual surface involvement. The majority of the caries development involved only a few patients, suggesting that perhaps only select patients undergoing fixed orthodontic treatment will have an increased caries risk (Southard, Cohen et al., 1986).

**Brackets and bands**

During orthodontic treatment, it has been found that the placement of bands and brackets on teeth influences the microflora of the mouth (Sakamaki and Bahn, 1968; Demling, Heuer et al., 2009). It has been shown that the presence of orthodontic appliances increases the salivary lactobacilli count (Owen, 1949; Bloom and Brown, 1964) as well as increases in staphylococci, streptococci, veillonellae, actinomyces and oral yeasts (Bloom and Brown, 1964; Kim, 2009; Costa, 2010). The increase in caries incidence in patients undergoing orthodontic treatment was first discussed by Noyes, who reported an average of 2.65 carious lesions per child pre-treatment and an average of 3.75 carious lesions after treatment (Noyes, 1937). Bach (1954) found a 5% increase in demineralisation and Ingerval (1962) found that the higher frequency of caries and precarious lesions in patients undergoing orthodontic treatment to be statistically significant at the 5% level. The placement of orthodontic bands has been shown to increase the levels of lactobacilli by five times (Sakamaki and Bahn, 1968). This count dropped back to the pre-treatment level once the bands were removed. This
showed that the increase in bacterial numbers was directly related to the placement of bands. The occlusal surfaces of the teeth, which are not directly affected by the presence of bands, also had increased levels of lactobacilli. This would tend to indicate that there is a possible increased susceptibility to caries on all exposed surfaces. The band edges might also act like grooves in the teeth thus providing a place for bacterial growth. The gingival edge of the bands showed twice the bacterial index of the occlusal edges, which are easier to clean with the toothbrush and via self-cleansing (Sakamaki and Bahn, 1968).

**Cements**

Different cements used to bond orthodontic brackets and bands may affect bacterial adhesion and demineralisation. A recent study compared three non-fluoride releasing composite resins; one fluoride releasing composite resin, one polyacid composite resin (compomer) and two resin-modified glass ionomer cements (Ahn, Lim *et al.*, 2010). Adhesion assays were performed by incubating each material with tritium-labeled cariogenic streptococci. While there was no difference in bacterial adhesion between the composite resins or compomer, streptococci adhered to the resin-modified glass ionomer cement at a significant level (Ahn, Lim *et al.*, 2010). This could potentially lead to a higher risk of caries development around brackets.
Plaque and bacterial numbers

Brackets and wires increase bacterial counts in the mouth. They serve as a place for food lodgement, and also interfere with the self-cleansing system of the mouth as they prevent the lips and cheeks from reaching the tooth surfaces and prevent the cleansing action of coarse foods during mastication (Boyd, 1983; Yoo, Park et al., 2007; Edith, 2010). An almost linear correlation between plaque and development of carious lesions has been demonstrated in orthodontic patients (Ogaard, 1989). Plaque acidity also tends to rise during orthodontic treatment (Edith, 2010). Pellegrini et al. (2009) studied plaque accumulation in self-ligating brackets vs brackets with conventional elastomeric ligatures. It was found that teeth bonded with self-ligating attachments had fewer bacteria in plaque than did teeth bonded with elastomeric brackets. At 1 and 5 weeks after bonding, the means for self-ligating vs elastomeric brackets were statistically lower for total bacteria and oral streptococci (Pellegrini, Sauerwein et al., 2009). Kitada et al (2009) found that numbers of opportunistic bacteria and fungi, as well as normal oral flora, had the ability to adhere to and colonize orthodontic brackets. Orthodontic patients, therefore, had higher numbers of bacteria in the mouth (Kitada, de Toledo et al., 2009). More recently, caries risk was assessed on patients who underwent orthodontic treatment with sectional brackets (Sanpei, Endo et al., 2010). The patients were categorized into high and low risk groups, based on pre-treatment bacterial counts, and were assessed both during and after orthodontic treatment. It was concluded that while there was a significant difference in bacterial counts between the two groups over time, Streptococcus mutans levels remained the same for both groups and only lactobacilli levels in the high risk group
increased during treatment. After treatment, however, bacterial levels returned to pre-treatment levels.

**Distribution of teeth affected**

Gorelick (1982), studied 121 patients who were undergoing fixed orthodontic treatment. It was found that of the patient sample, 49.6% had white spot formation on at least one tooth (Gorelick, Geiger et al., 1982). The highest percentage of incidence was found on the maxillary anterior, 15.3%, and mandibular posterior segments, 14.1%. The mandibular anterior and maxillary posterior segments showed lower percentage of 9.4% and 4.5%, respectively (Gorelick, Geiger et al., 1982). The highest incidence was found on the maxillary lateral incisors at 23%. The maxillary central incisors were at almost three times less risk to develop decalcification (8.4%). Decalcifications are more prevalent on the cervical and middle thirds of the teeth (Mizrahi, 1983). No significant difference was seen between right and left sides of the arches (Benson, Pender et al., 1999). Maxillary incisors that had been banded as opposed to bonded also showed an increase in decalcification when compared to the control group. Again, the lateral incisors showed the highest rate of 17%, close to two and one half times that of the central incisors. This is similar to the bonded group with both groups showing significantly higher decalcification development than the control group (Gorelick, Geiger et al., 1982).

The increased incidence of white spot development on lateral incisors when compared to central incisors may suggest that the small tooth surface area between the gingival and bracket margins increases plaque retention and
makes oral hygiene more difficult. As 50% of the treated sample had no white spot development, the potential influence of individual differences in enamel structure, composition of saliva, oral hygiene and other variables seems to be an important consideration in determining the patient’s risk of decalcification during treatment.

**Post orthodontic treatment analysis**

Al Mulla *et al*, 2009, examined caries-related factors shortly after the completion of orthodontic treatment and then used the Cariogram computer program to describe caries risk profiles at follow-up in these patients (Al Mulla, Kharsa *et al*., 2009). All patients were examined after debonding in the following order: interview, plaque score, caries examination, saliva samples, bitewing radiographs, panoramic radiographs, and intra-oral digital photos. Carious lesions in both the enamel and dentine were diagnosed clinically and radiographically and were included in the decayed and filled surfaces (DFS) index. A paraffin-stimulated whole saliva sample was collected for estimations of secretion rate, buffer capacity, and number of mutans streptococci (MS) and lactobacilli (LB). When evaluating the nine caries-risk indicators, three appeared to be of great importance; i.e., DFS, MS, and LB. The number of DFS the patient has before orthodontic treatment is a strong predictive factor of his/her caries risk. When checking the mutans streptococci and lactobacilli scores, both appear to have a strong effect on DFS and the caries risk. The importance of plaque amount as a factor for estimating caries risk was, however, shown to have a fairly weak association with caries.
**Multifactorial aetiology**

Several authors have discussed multiple factors in relation to orthodontic treatment and caries risk; caries development, plaque accumulation, effect of fluoride, and demineralization. The complexity of the issue is highlighted and it is generally concluded that there is not one single factor explaining the changes observed. Thus, in one and the same population, different explanations may be relevant for different individuals, for different age groups, for different teeth and for different periods of time (Bratthall, 1996).

**Oral flora associated with dental caries**

The bacterial species associated as being the main caries causing odontopathogens in humans include *Streptococcus mutans*, *Streptococcus sobrinus* and members of the Lactobacillus family (Bowden, 1997). Other species, however, including Actinomyces species, have also been proposed to play roles in the caries development process (Bowden, Ekstrand et al., 1990; Nyvad and Kilian, 1990; van Houte, 1994; Schupbach, Osterwalder et al., 1995). It can, therefore, be concluded that, in any individual, the processes which lead to caries at specific sites varies over time and also in terms of the dominant bacterial species (Bowden, 1997).

It is hoped that this variance can be indentified in an individual by a microbiological analysis of saliva taken from the mouth. However, analysis of
saliva will not show the oral distribution of the species of bacteria or whether it is present in a high concentration on one tooth surface or low concentrations on several sites but it can give an indication of the general number and type of bacteria in the individual's oral cavity (van Houte, 1993).

**Diversity of micro-organisms involved in dental caries**

Analyses of saliva samples related to caries activity have focused mainly on species which have strong associations with the disease process (Beighton, 1991; van Houte, 1993; Bowden, 1997). This has had the potential to limit consideration of other organisms involved in caries (Beighton, 2005). One paper looked at standardized samples of plaque associated with root caries, and they showed the diversity of the species found over sound, carious and arrested lesions (Schupbach, Osterwalder et al., 1995). They proposed Prevotella spp, and Capnoeytophaga spp, in the breakdown of cementum and dentine and found no association between lesions and mutans streptococci, Lactobacillus spp, or *Streptococcus mitis*. These results do not agree with other authors, who found that mutans streptococci and Lactobacillus species could be implicated in root caries (Bowden, Ekstrand et al., 1990; van Houte, 1994). The difference in results may be due to differences among individuals and or sampled sites.

**Historical and current nomenclature of mutans streptococci**

*Streptococcus mutans* was first described by Clark after he was able to isolate it from a carious lesion (Clark, 1924). Many years later, when the interest into
dental caries increased, further research revealed the presence of many strains which were biochemically very similar (Whiley, Beighton, 1998). Altogether, 7 serotypes were described, these were classified as a-g.

Further research into the protein profiles of the strains, their cell wall structures and DNA composition revealed that there was considerable variation in the isolates identified as *Streptococcus mutans*.

On the basis of this research, the individual serotypes were classified into distinct species (Whiley, Beighton, 1998). The name mutans streptococci was used to describe this whole group of streptococci species, and the name *Streptococcus mutans* was retained to describe the most common of the human strains, with the other being *Streptococcus sobrinus*.

In this thesis, mutans streptococci will be discussed most commonly, which encompasses both human cariogenic strains of oral streptococci, *Streptococcus mutans* and *Streptococcus sobrinus*. Occasionally there will be reference to *Streptococcus mutans*, which relates to the individual strain of bacteria.

**Mutans streptococci**

Mutans streptococci are especially important as they are associated with the initial development of carious lesions (van Houte, 1994). Of the mutans streptococci group, *Streptococcus mutans* and *Streptococcus sobrinus* are the most commonly isolated from humans (Chestnutt, MacFarlane *et al.*, 1994). Lactobacilli, *Streptococcus mutans* and *Streptococcus sobrinus* are all linked to
the development of dental caries (Street, Goldner et al., 1976; Winter, 1990; van Houte, 1994).

Mutans streptococci, which are Gram positive and found at high levels in saliva and plaque, are associated with a high caries incidence. High levels can also be isolated from carious lesions. They are both acidogenic and aciduric, and are facultative anaerobes (Denepitiya and Kleinberg, 1984). Streptococci are the major acid producers in dental plaque (Iwami, Hata et al., 1989). *Streptococcus mutans* can metabolise a wider variety of carbohydrates than any other Gram positive micro-organisms indentified so far (Mitchell, 2003). Genes for the transport and metabolism for glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, β-glucosides, trehalose, maltose, raffinose, ribulose, melibiose, starch, isomaltosaccharides and possibly sorbose are present in its genome (Mitchell, 2003). The glycolytic pathway leads to the production of pyruvate which in turn is reduced to other acids (Mitchell, 2003) (figure 1). Under aerobic conditions, lactic acid is produced almost exclusively, with smaller amounts of formic and acetic acids (Abbe, Takahashi et al., 1982; Iwami, Hata et al., 1989; Jacobson, Lodge et al., 1989).

Under anaerobic conditions, lactic as well as more formic and acetic acids are produced (Abbe, Takahashi et al., 1982; Iwami, Hata et al., 1989). In the presence of excess fermentable carbohydrates, lactic acid is rapidly produced almost exclusively (Duguid, 1985).

The main methods to analyse acid production described in the literature are alkaline titration (Iwami and Yamada, 1985), colorimetry (Iwami and Yamada, 1980), gas chromatography (Carlsson, 1973), and isotachophoresis (Geddes, 1972).
Figure 1. The main virulence factor of *Streptococcus mutans* is the production of acid as part of the bacterial biofilm that constitutes dental plaque. *Streptococcus mutans* also expresses a variety of cell-surface and secreted virulence factors, including a haemolysin (Mitchell, 2003).

**Lactobacilli**

Lactobacilli are Gram positive rods which occur naturally on vegetation and fruits, and in the normal flora of the human body. They have the ability to use carbohydrates to produce large amounts of lactic acid. They are both acidogenic (produce acid) and aciduric (tolerate acid environments), and can grow in both aerobic (presence of oxygen) and anaerobic (absence of oxygen) conditions (Badet and Thebaud, 2008).

Lactobacilli were one of the first families of bacteria to be associated with dental caries (Owen, 1949). The presence of it in the oral cavity varies between studies, with 40% to 100% of children over the age of 2 years having lactobacilli being reported to be part of their commensal oral flora (Kohler,
Andreen et al., 1984; Klock and Krasse, 1987) according to Badet and Thebaud (2008). The association between lactobacilli and dental caries was clearly established in the 1950’s (Badet and Thebaud, 2008); however, further research into this association has indicated that lactobacilli are present in established lesions, rather than early demineralisation (Boyar and Bowden, 1985; Tanzer, Livingston et al., 2001); that is they are not the primary initiators of dental caries.

**Other cariogenic bacteria**

There are studies that have shown that early enamel caries will occur where mutans streptococci are absent or below the level of detection (Loesche and Straffon, 1979; Kristoffersen, 1985; Lang, Hotz et al., 1987; Marsh, Featherstone et al., 1989). While these are relatively rarely documented, model systems have also shown the presence of early enamel demineralization without detecting *Streptococcus mutans* (Boyar, Thylstrup et al, 1989; Macpherson, MacFarlane et al., 1990). This must suggest that early caries development can be produced, under certain conditions by organisms other than mutans streptococci (Bowden, 1997). The exact nature of these organisms is not well documented, although they have the ability to produce low pH levels, and resemble *Streptococcus anginosus*, *Streptococcus mitis*, *Streptococcus gordonii* and *Streptococcus oralis* (Sansone, van Houte et al., 1993; van Houte, 1994).
It can be seen that samples from an individual may include a range of bacterial species that could be considered to be involved in the initiation and progression of dental caries and also could be used as indicators of cariogenic activity (van Houte, 1993; van Houte, 1994). In addition to species diversity, variation among strains of a species will also contribute to a pattern of the flora unique to an individual (Bowden, 1997).

Variation within individual microbial species

It has been reported that bacterial strains within a species can show wide variation of characteristics, and can often be difficult to accurately define. Methods that have shown this include; serotyping (Masuda, Tsutsumi et al, 1979; Beighton, Rippon et al, 1987), bacteriocin typing (Davey and Rogers, 1984) and biotyping (Kilian, 1989) according to Bowden (1997). Mutans streptococci also show variation within the species (Kohler and Krasse, 1990; de Soet, van Loveren et al., 1991; Macpherson, MacFarlane et al., 1992). Some strains are more virulent than others. The presence of a particular strain of bacteria, i.e. virulent and/or avirulent, could influence the caries outcome of the individual.

One of the most important aspects in the identification of variants of pathogenic bacteria is that, in some individuals, this can be associated with virulence and, therefore, possible caries risk. It may be possible that specific subgroups of a
species have their own unique function within the oral environment. In the resident oral flora of humans, several subgroups of the same species may exist within one environment (Bowden, 1997). It has been reported that the existence of many variants of a species is typical of both commensal organisms and/or opportunistic pathogens (Loos, Dyer et al., 1993).

**Mutans streptococci**

Mutans streptococci have many strains, some of which have a much higher capacity for acid production, and thus are a greater risk for the development of dental caries. Studies have shown that an individual tends to acquire one or more consistent strains of the bacteria from a relatively young age. Some individuals gain and/or lose strains, thus changing their caries risk (Lindquist and Emilson, 2004). Many studies have shown that the levels of these bacteria in saliva correlate to the caries risk of a patient (Bowden, 1997; Tenovuo, 1997; Campus, Lumbau et al., 2000).

It has also been shown that patients can be colonized with more than one type of species of *Streptococcus mutans* (Caufield and Walker, 1989; Alaluusua, Alaluusua et al., 1994; Alaluusua, Matto et al., 1996). The possibilities of measuring genetic subtypes of *Streptococcus mutans* include; looking at species diversity; determining the stability of individual variants in a person; monitoring changes in subgroups over time; and detecting any variants specifically associated with caries (Caufield and Walker, 1989). This would potentially highlight the individuality of the microflora of a subject, monitoring variations in the state of the organism and possibly its association with caries.
Many individuals can harbour a number of genotypes of a bacterial species in the oral cavity and these can be quite different from person to person. It may be possible that all these species are very similar; therefore, they survive together and perform the same function (Bowden, 1997).

There are very few studies of commensal oral bacteria which relate specifically to individual clones. One such study looks at the genetic types of *Streptococcus mitis biovar 1*, and it showed 24 different genotypes among 106 isolates from a mother, father and child (Hohwy and Kilian, 1995). They all had similar clones, but each individual did show their own genotypes in some of the species. Analysis of these clones also showed variation in carbohydrate antigens. Of the 24 genotypes isolated, 12 serotypes were also identified (Hohwy and Kilian, 1995). Yoo, Parks *et al.* (2007) looked at the biotype of mutans streptococci and found 95 strains in 141 subjects. 77 of these strains were *Streptococcus mutans* and 18 were *Streptococcus sobrinus* (Yoo, Parks *et al.*, 2007).

**Lactobacilli**

The lactobacilli group of bacteria contains over 80 different species, not all of them found in the oral cavity (Badet and Thebaud, 2008). They can all be divided phylogenetically into three separate groups. These different species also show morphological and physiological differences.

In saliva samples of subjects with dental caries, seventeen different species have been identified as being present (Smith, Aweh *et al.*, 2001; Teanpaisan and Dahlen, 2006; Badet and Thebaud, 2008). In plaque samples, sixteen species have been identified, with three species being different to those found
in saliva (Badet and Thebaud, 2008). This may be due to different adherence factors present in the individual species.

Caries Prediction

“There are three basic prerequisites for a successful application of the high-risk strategy in controlling dental caries. First, the occurrence of caries in the target population must be low enough to justify the effort and expense of identifying individuals who are believed will develop an unacceptably high number of cavities. Second, one must have accurate, acceptable and feasible measures for identifying the subjects with an unacceptably high risk. Third, the preventive efforts that aim at bringing down the elevated risk of these subjects should be based on measures that are effective and feasible (Hausen, 1997)”.

The majority of children and adolescents in recent times have very few, if any, cavitated lesions; however, there is still a small group of the population where the caries rate remains high (Hausen, 1997). It would be ideal if these patients could be identified before the development of dental caries so a preventive treatment program can be put into place. In order to be able to make an individual risk assessment for the patient, we need to know what happens to individuals with different levels of various risk factors and their combinations if no preventive treatment was offered to them.
**Prediction studies**

When looking at prediction studies there are two dichotomies: that is, the individuals for whom it is believed that the risk is high vs. low and the individuals for whom it is observed a true high vs. low caries increment.

There are four groups within these two dichotomies. The first group consists of correctly classified individuals, true positives, for whom it was believed that the risk was high and whose actual caries rate was high. The fourth group represents correctly classified true negatives. For individuals falling into groups two and three, misclassification has occurred. For the false positives in group two, a high risk is assumed but the true caries increment is low. Correspondingly, the false negatives in group three are believed to have a low risk, but their actual caries increment is high.

**Predictors**

In practice, several predictors are often taken into consideration in prediction studies. In the case of multiple predictors, each of them can be considered separately, which leads to predictor-specific numbers of true and false positives, and true and false negatives. On the contrary, the information of many predictors can be condensed into a single variable on the level of which the prediction of high vs. low risk is based. The techniques for this kind of condensing range from combinations of two variables, e.g. mutans streptococci count and lactobacilli count, to regression-based multivariable models. Even in the case of one predictor, the risk markers are seldom natural dichotomies (Hausen, 1997).
Accuracy of caries prediction

A perfect risk marker has a sensitivity of 100%, and a specificity of 100% thereby implying there are no errors in risk assessment. Consequently, the false positive rate and false negative rate are 0% with the positive and negative predictive values being 100%. Having perfect accuracy means that the predicted high-risk group would consist of true high-risk individuals only and that only true low-risk individuals would be included in the predicted low-risk group. Unfortunately, no such marker is presently available for the assessment of caries risk. Therefore, a certain proportion of errors must be accepted. It has been suggested that in a risk model, the sum of sensitivity and specificity
should be at least 160% before a caries risk marker can be considered accurate enough to be considered for targeting individualized prevention (Kingman, 1990). This agrees with an alternative suggestion (Wilson, 1989) which reported that a sensitivity and specificity of 80% each would be acceptable for practical use in the community. While neither of these suggestions takes into account the fact that errors related to poor sensitivity have consequences that are different from those related to poor specificity, they are a potential starting point in evaluating the performance of proposed markers for high caries risk.

**Sensitivity and specificity in a clinical setting**

What would a combined sensitivity and specificity of 160% mean in a clinical setting? If both the sensitivity and specificity were 80%, every fifth patient with a true high risk would remain undetected in a risk assessment and, therefore, would not receive the preventive treatment against caries that they may need. Also, every fifth patient with a true low risk would be included in the high-risk group and receive preventive measures to no or little purpose (Hausen, 1997), thus wasting resources and increasing cost. Therefore, even the proposed minimum acceptable level of accuracy would seem to result in a high rate of classifications in the wrong category.
Mutans streptococci and lactobacilli as caries predictors

It is quite a complex matter to determine the suitability of counts of lactobacilli and mutans streptococci for the assessment of caries risk. Different standards apply for different groups. For example, the prediction of the caries increment for caries-free children on an individual basis and the identification of high-caries-risk groups for the purpose of instituting preventive measures are quite different (van Houte, 1993). There is a large amount of literature available and this consists of a variety of cross-sectional and longitudinal studies. The interpretation is often difficult as they involve different subject populations (age, caries experience, etc.), different sample sources (plaque, saliva) and sampling methods, and different methods for bacterial enumeration.

The presence of lactobacilli and mutans streptococci in plaque on sound, as well as carious enamel, and the frequent absence of lactobacilli, particularly in plaque associated with early carious lesions suggest a limitation of their usefulness as caries-risk predictors (van Houte, 1993). This has been documented by a variety of longitudinal studies involving either conventional laboratory procedures for the enumeration of these organisms in plaque or saliva for the Snyder test (Snyder, 1940; Becks, 1944; Davies, 1959; Snyder, 1963). The Snyder test measures the time required for the lactobacilli in the saliva test sample to produce a degree of acidity which causes a particular colour change of the pH indicator (Snyder, 1940). The Snyder test will be discussed in more detail in the following section looking at tests based on bacterial acid production.
There are other studies that have shown that lactobacilli counts are positively correlated with caries activity (Crossner, 1981; Kingman, 1988; Alaluusua, 1990; Russell, MacFarlane et al., 1991). It would appear, therefore, that the reliable prediction of caries risk by means of lactobacilli counts for individuals is not possible if the acceptable correlation coefficient is taken to be in the range of 0.9 to 1.0 (Socransky, 1968).

Table 1 shows data on sensitivity (the capacity of an organism or sense organ to respond to stimulation), specificity (the condition of being peculiar to a particular individual or group of organisms and predictive values (http://www.nlm.nih.gov/medlineplus/mplusdictionary.html, 2009).

Some of the studies provide details on the correlation between increasing levels of lactobacilli or mutans streptococci and caries incidence (Crossner, 1981; Newbrun, 1984; Kingman, 1988; Alaluusua, 1990) and these studies along with many others confirm the positive association between counts of lactobacilli or mutans streptococci and caries activity.
<table>
<thead>
<tr>
<th>Authors</th>
<th>Sample Size (N)</th>
<th>Subject Age (Yr)</th>
<th>Caries Evaluation</th>
<th>Sampling/Enumeration</th>
<th>Screening/Criterion</th>
<th>Validation Criterion</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>Predictive Value +%</th>
<th>Predictive Value -%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alaluusa et al., 1990</td>
<td>122</td>
<td>12-17</td>
<td>Incipient Lesion (L) excluded</td>
<td>Saliva; dip-slide 3 samples</td>
<td>MS&gt;10^5 CFU/mL L&gt;10^5 CFU/mL</td>
<td>3 year caries incidence, &gt;3% of surfaces at risk</td>
<td>29</td>
<td>88</td>
<td>45</td>
<td>78</td>
</tr>
<tr>
<td>Crossner, 1981</td>
<td>107</td>
<td>14.5</td>
<td>Incipient L</td>
<td>Saliva; dip-slide 7 samples</td>
<td>L&gt;10^5 CFU/mL</td>
<td>64 week caries incidence, 2 or more lesions</td>
<td>56</td>
<td>92</td>
<td>76</td>
<td>82</td>
</tr>
<tr>
<td>Kingman et al., 1988</td>
<td>541</td>
<td>10-15</td>
<td>Incipient L</td>
<td>Saliva; MS (MSB agar), L (Rogosa agar) 2 samples</td>
<td>MS&gt;10^5 CFU/mL L&gt;10^5 CFU/mL MS&gt;10^2 CFU/mL</td>
<td>17 mth incidence &gt;3 new DMFS</td>
<td>5</td>
<td>98</td>
<td>38</td>
<td>75</td>
</tr>
<tr>
<td>Russell et al., 1991</td>
<td>372</td>
<td>Adolescents</td>
<td>Incipient L</td>
<td>Saliva; MS (MSB agar), L (Snyder Test, Rogosa agar) 1 sample</td>
<td>MS&gt;10^5 CFU/mL L&gt;10^5 CFU/mL</td>
<td>2 year caries incidence &gt;4.4 new surfaces</td>
<td>48</td>
<td>68</td>
<td>47</td>
<td>68</td>
</tr>
<tr>
<td>Wilson and Ashley, 1989</td>
<td>84</td>
<td>11-12</td>
<td>Incipient L excluded</td>
<td>Saliva; MS (TYCSB) L (dip-slide) 1 sample</td>
<td>MS&gt;10^5 CFU/mL L&gt;10^5 CFU/mL</td>
<td>3 yr caries incidence 8 or more DFS</td>
<td>79</td>
<td>53</td>
<td>-</td>
<td>59</td>
</tr>
</tbody>
</table>

Table 1. Sensitivity, Specificity, positive and negative predictive values for different types of studies with lactobacilli and/or mutans streptococci as caries-risk predictors (van Houte 1993)
Variation in prediction

The prediction of future caries risk on the basis of different levels of the indicator organisms alone is difficult. In many studies, the validation level is selected so as to enable evaluation of predictions for a high-caries-risk group. These groups usually comprise of about 25% of the subjects (Wilson, 1989; Alaluusua, 1990) and are composed of subjects with a caries incidence above the mean (Crossner, 1981; Russell, MacFarlane et al., 1991). Overall, the data tend to indicate that the prediction of high caries risk in children, on the basis of a single microbial factor is difficult, whereas the prediction of low caries risk is more reliable.

Many factors other than caries prevalence may also influence the test results and contribute to the wide variation of the predictive values among studies. Subject age may be an important variable, since subjects at various ages may have different proportions of newly-erupted teeth which are more prone to caries than longer-erupted teeth (Carlos, 1965).

Caries evaluation has many difficulties. The methods used vary greatly and in general, methods can be a major source of error. For example, the use of saliva has been questioned because it contains bacteria derived not only from the teeth but from other mucosal surfaces as well. The concentrations of mutans streptococci in saliva may also vary considerably, due to toothbrushing or methodological errors (Togelius, 1984). Preferably, saliva should be sampled at the same time of the day to reduce error. Also, saliva sampling doesn’t show whether the levels of lactobacilli and mutans streptococci reflect a few highly-populated dentition sites or many sparsely-populated sites, whether they reflect
cells in sound or carious tooth surface areas, or where the bacteria are localized in the dentition (Krasse, 1988).

Several studies, however, have shown a good positive correlation between the concentrations of mutans streptococci in saliva and the prevalence of mutans streptococci on different tooth surfaces (Duchin, 1978; Togelius, 1984; Keene, 1986; Lindquist, 1989), but these findings do not exclude the possibility that caries may develop on one or a few mutans streptococci-infected sites, while saliva samples indicate a very low bacterial level.

However, the results of nearly all the longitudinal studies shown in Table 1 are based on a saliva sampling frequency of one or less per year. In view of potential short-term variation in the sample concentrations of mutans streptococci, that is the possibility of short-term changes in dietary habits or other factors, an increased sampling frequency is likely to be more accurate. Some studies (Pienihakken, 1988) suggest that sampling more than two-three times per year may improve caries-risk prediction.

**Saliva Testing**

Each individual has unique characteristics which will influence their risk of developing dental caries. However, the underlying microflora is similar among different people. While there are variations at a species and sub-species level, this does not necessarily mean that the characteristics that will be expressed
are different (Bowden, 1997). While microbiological tests cannot be the only measure of caries potential, they can be one important consideration in determining a patients’ potential for developing the disease.

**Tests based on culturing and bacterial numbers**

Due to the implications of lactobacilli and mutans streptococci in the development of dental caries, predictive tests for caries risk focus on the enumeration of these bacteria. Some of the available chair-side tests today focus on culturing and growing bacteria taken from a saliva sample.

**Dip-Slide test**

One of the most basic of tests involves sampling the individual’s saliva. The Dip-Slide Test (Dentocult SM®) is produced by Orion Diagnostics, Finland, and is a simple test which can be performed with minimal training (Jordan, Laraway *et al*., 1987). Stimulated saliva is collected from the patient and samples are placed onto the culture media. Mutans streptococci sampling is carried out on mitis-salivarius-bacitracin (MSB) media, and lactobacilli on selective agar strips. After incubation at 37°C for 48 hours, the colonies can simply be counted. Counts greater than $10^6$ colony forming units (CFU) per ml of mutans streptococci are indicative of a high caries risk, while less than 100000 CFU/ml is considered a low caries risk (van Houte, 1993; Samaranayake, 2002). A similar dip test was manufactured by APO Diagnostics, Canada, and is called Cariescreen SM®. For this test a bacitracin tablet is dissolved in a vial
containing a buffered saline. Stimulated saliva is then collected in this same vial. The test (dip-slide) which is a modified MSB agar (minus bacitracin) is then briefly exposed in this diluted saliva. A CO₂-generating tablet is then placed into the vial; the screw cap dip-slide is closed in the vial, and incubated for 48 hours at 37 °C. Colony numbers are then compared to a reference density chart. Growth of reference strains for the Cariescreen SM® are similar to culturing on MSB agar plates (Jordan, Laraway et al., 1987). There have been many variations of the dip test. These tests all have very similar methods and materials and all show similar accuracy in the capacity to predict high vs low risk individuals (Pinelli, Serra et al., 2001; Thaweboon, Thaweboon et al., 2006; Sanchez-Garcia, Gutierrez-Venegas et al., 2008).

Limitations

While these saliva tests are simple to perform they take two days to incubate and culture the samples. Also, testing has shown that traditional culture based media, such as MSB agar, demonstrate less support for bacterial growth than trypticase-yeast extract-cysteine-sucrose-bacitracin (TYCSB) agar (Hildebrandt and Bretz, 2006). Some species of mutans streptococci have the potential to not grow on these test media, hence all the cariogenic bacteria may not be included in the results leaving them inaccurate. The frequency of isolations of pure cultures on TYCSB agar was 91-97% compared to only 37% for MSB medium, 47% for Cariescreen SM® and 69% for Dentocult SM® (Hildebrandt and Bretz, 2006). The addition of not only bacitracin, but also sucrose to TYCSB media acts to inhibit the growth of most commonly recovered bacteria
other than mutans streptococci (Hildebrandt and Bretz, 2006). Sucrose aids in the growth of mutans streptococci and produces colonies with a specific morphology, due to the synthesis of extracellular polysaccharides. This can make identification of the colonies much easier.

**Colour Indicators**

A quicker method for testing *Streptococcus mutans* numbers is available and called GC Saliva-Check Mutans®. It is claimed to be a 15 minute test to determine if the patient has a resting level of more than $10^5$ *Streptococcus mutans* in their saliva. The test uses species specific monoclonal antibodies to detect the *Streptococcus mutans* in the saliva sample (Matsumoto, Sugihara et al., 2006). Initially the patient chews on some paraffin wax to gain stimulated saliva. The saliva is collected and mixed with one drop of reagent (provided as part of the kit). Four drops of a second fluid are then added and once the saliva turns a greenish colour, a pipette is used to place three drops of saliva onto the test carrier. After 15 minutes, if the sample window in the test carrier shows a pink-red strip, the patient has more than $10^5$ CFU/ml of saliva, and an increased caries risk. The test carrier also has a control window which must always show a coloured strip to show the test is working.

**Limitations**

While an independent study (Matsumoto, Sugihara et al., 2006) has shown this test to correlate well to counts grown on MSB agar plates, it concluded that, as
the test only identifies *Streptococcus mutans* and not species of *Streptococcus sobrinus*, another species of cariogenic bacteria in saliva, there was a potential to miss some patients who are high risk (Matsumoto, Sugihara *et al.*, 2006). The study also reported that the Dentocult SM® test provided a higher correlation in identifying potential cariogenic bacteria in the mutans streptococci family. It is also interesting that the GC saliva-check Mutans ® uses $10^5$ CFU/ml as a level for high caries risk, when most other papers report that $10^6$ CFU/ml indicates a high risk for caries (Kingman, 1988; Wilson, 1989; Alaluusua, 1990; Russell, MacFarlane, 1991).

**Historical tests**

Other techniques which have been used in the past include the use of wooden spatulas, moistened with saliva and pressed into selective media for *Streptococcus mutans* (Kohler and Bratthall, 1979) and selective medium-colour indicator tests for use in epidemiological surveys for the presence or absence of *Streptococcus mutans* (Jordan, Laraway *et al.*, 1987). These tests have proven useful in identifying low risk patients, but were unable to correlate as well to high caries levels (Jordan, Laraway *et al.*, 1987).

**Tests based on acid production**

These saliva tests measure the production of acid by the micorflora present in the oral cavity and use this as a guide to caries risk. Many of these are non-
specific to the type of bacteria tested. Some tests use an acidic medium to select for the aciduric species that tolerate the environment.

**Colour indicators**

A new chair-side test has recently been developed that measures lactic acid production of cariogenic bacteria in plaque (Bretz, Corby *et al.*, 2007; Chaussain, Opsahl Vital *et al.*, 2010). This test uses a bacterial sample taken from the patients’ tongue, using a stick with a cotton swap impregnated with sucrose. The swap is then placed into the Clinpro Cario L-pop blister, which is then activated to promote a chemical reaction. There is a colour change within 5 minutes, which is then compared to a colour reference chart provided with the test (Chaussain, Opsahl Vital *et al.*, 2010). The risk of dental caries is rated according to a scale; low (1-3), moderate (4-6), or high (7-9). The test has been used to predict caries development among adolescent orthodontic patients (Chaussain, Opsahl Vital *et al.*, 2010), and also to test against current decay rates among infants and children (Bretz, Corby *et al.*, 2007).

**Historical tests**

The Rickles Test (Rickles, 1953) and the Snyder Test (Birkhed, Edwardsson *et al.*, 1981) are two tests which place the salivary microorganisms (mainly lactobacillus) into a fermentable carbohydrate medium (such as sucrose) with indicators which change colour over a period of time according to the level of acid produced.
Rickles (1953) described the measurement of dental caries activity by looking at 3 different tests; a new colorimetric pH determination, total titratable acid, and the *Lactobacillus acidophilus* index (numbers) (Rickles, 1953). He concluded that while the three tests each correlated well with dental decay development and with each other, the pH colorimetric test was the most simple to use and would be the most likely test to be used as a chair-side saliva test for decay. The Rickles test requires incubation at 37°C for four hours and colour change is sensitive to levels of 0.4 pH intervals (Rickles, 1953).

The Snyder test involves saliva being cultured with Snyder test agar (Difco Laboratories) in a screw-capped bottle. After incubation for up to 72 hours, colour change is assessed. A positive colour change in 24 hours indicates high caries activity, positive colour change in 48 hours indicates moderate (average) caries activity, negative colour change in 48 hours indicates slight caries activity, and negative colour change in 72 hours indicates negative caries activity (Snyder and Clarke, 1950).

**Limitations**

The Snyder test is mainly used to test acid production from lactobacilli (Snyder, 1940). The Snyder test can be difficult to read accurately as the whole agar does not change colour, only parts, which may lead to inaccuracies and the test, while simple to perform, requires some equipment not readily available in the dental clinic (Sims, 1968). Various modifications to the Snyder test have been made in the past with changes being made to the concentration to the
colour indicator to make the end result easier to read and, therefore, more accurate (Sims, 1968).

Testing of infants and children with Clinpro Cario L-pop revealed the test was useful in explaining levels of caries; however, 23% of the subjects were caries-free during testing and the test indicated only 7% of subjects showed a low production of lactic acid (Chaussain, Opsahl Vital et al., 2010). This tends to over-estimate the prevalence of dental caries in a population. Among the orthodontic patients, with this test being used as an indicator for caries risk, 80% were categorised as having a high risk of caries by the end of the study (Chaussain, Opsahl Vital et al., 2010). Only 54 of the 110 subjects actually developed at least one carious lesion, showing that this test tends to over-categorise patients into a high risk group.

Other parameters measured in saliva tests

Calcium and Phosphate ions

Aiuchi, Kitasako et al., (2008) used stimulated saliva to compare buffer capacity and dissolved calcium and phosphate ions. These samples were titrated with a solution of hydrochloric acid (HCL) and pH was measured. As the enamel dissolved, both the pH and the concentrations of calcium and phosphate increased until the dissolution of hydroxyapatite occured (Aiuchi, Kitasako et al., 2008). In this study, different levels of buffer capacity appeared to show variation in enamel dissolution that could be used as an indicator of the cariogenic potential. A high saliva buffer capacity may result in an increased
surface pH of the enamel crystal, resulting in favourable conditions for mineral uptake and, thus, remineralization.

While this method has the potential to be used as a diagnostic tool for high risk individuals, this study didn’t correlate the findings with caries rates, so the sensitivity and specificity of the test have not been reported. Also, the materials required for this test appear not to be standard equipment for the dental office.

**Oligosaccharides**

Salivary glycoproteins contain individually specific oligosaccharide motifs. Depending on microbial compatibilities and individual genotypes, the glycoproteins that form the pellicle coating of teeth may provide attachment sites that foster colonization leading to cariogenesis (Denny, Denny et al., 2007). Resting saliva samples were taken and oligosaccharide moieties were quantitated in dried spots of saliva on nitrocellulose. A combination of multiple linear regression and neural net analyses were used to develop the algorithms that describe the relationship between oligosaccharide patterns and decayed/filled teeth index (DFT) (Denny, Denny et al., 2007).

Both children and adults were looked at in this study. It was claimed that the adult sample showed an accurate correlation with oligosaccharides and past caries history (P<0.001 (Denny, Denny et al., 2007)).

This test is an interesting concept; however the method appears to be very technical and is unlikely to be developed into a simple chair-side test.
Conclusion

An ideal risk test would be quick, inexpensive, simple to use, require minimal or no training so anyone can conduct the test, and be accurate in predicting the caries risk of the patient.
References


Statement of Purpose

Hypothesis

It is hypothesised that the acid produced from the salivary microflora as a whole is an accurate way to predict patients at an increased risk of developing white spot lesions during fixed appliance orthodontic treatment. Therefore, it is intended that the proposed research evaluate a test based on the above hypothesis, which if successful, could be developed into a simple, quick and accurate clinical saliva test to determine if an individual is at an increased risk of developing enamel demineralisation.

Aims

1. To evaluate a test using the acid producing potential of bacteria in saliva as an indicator for risk of enamel demineralisation in the patient wearing fixed orthodontic appliances.

2. To compare patients who have been identified as both high and low risk by the test, and analyse their salivary mutans streptococci for differences in bacterial counts, metabolism and acid end product production.
SECTION 2

Article 1

This article has been written in the format for submission to the Journal of Dental Research.
Salivary pH change as a predictor of white spot lesion development in patients undergoing orthodontic treatment

(Journal of Dental Research)

Dr. Sara Roberts
School of Dentistry
The University of Adelaide
Adelaide
South Australia
5005

Professor Wayne Sampson
Begg Chair in Orthodontics
The University of Adelaide
Adelaide
South Australia
5005

Dr. Neville Gully
School of Dentistry
The University of Adelaide
Adelaide
South Australia
5005

Assoc. Professor Craig Dreyer
School of Dentistry
The University of Adelaide
Adelaide
South Australia
5005
Salivary pH change as a predictor of white spot lesion development in patients undergoing orthodontic treatment
Abstract

Introduction: Orthodontic braces pose risks to both hard and soft tissues, with development of demineralisation of enamel the most common (Travess, Roberts-Harry et al., 2004). An ideal saliva test would identify patients at an increased risk of developing this problem. Materials and Method: Fifty-two patients due to start fixed appliance orthodontic treatment agreed to participate. Saliva samples, collected before braces were placed and during treatment at six-eight week intervals, were mixed with a buffered solution containing sucrose (10% w/v), at pH 5.7, and rate of pH change was measured over 30 minutes. Demineralisation development was determined from standardized intra-oral photographs. Results: Of the fifty-two participants, three developed verifiable demineralisation. Two were identified as high risk from their saliva test, one as low risk, giving a sensitivity of 67%, and specificity of 94%. Conclusion: This technique has the potential to be developed into a commercial chair-side saliva test.
Introduction

Orthodontic treatment is a common occurrence with up to 29.7% of the adolescent population (Bolle, Cunha-Cruz et al., 2007) and 1% of the adult population (Whitesides, Pajewski et al., 2008) receiving fixed braces. This type of treatment poses significant risks to the hard and soft tissues. One of the most common complications of fixed orthodontic appliance treatment is the demineralization and subsequent white spot lesion development in the enamel (Travess, Roberts-Harry et al., 2004). White spot lesions are an early visible sign of dental caries and the incidence of white spot lesions in orthodontic patients has been reported as being as high as 50 per cent (Gorelick, Geiger et al., 1982; Lundsrtöm and Krasse, 1987; Lovrov, Hertrich et al., 2007) with white spot lesions sometimes occurring as early as one month after banding (Ogaard, Rølla et al., 1988).

It is commonly thought that the placement of a full fixed appliance makes the mechanical removal of plaque more difficult, therefore, increasing caries risk. Orthodontic bands and brackets influence the microflora of the mouth (Sakamaki and Bahn, 1968; Demling, Heuer et al., 2009) and have been shown to result in an increase the salivary lactobacilli count (Owen, 1949; Bloom and Brown, 1964), as well as staphylococci, streptococci, veillonellae, actinomyces and oral yeasts (Bloom and Brown, 1964; Kim, 2009; Costa, 2010).

Various tests use bacterial counts of cariogenic bacteria in saliva as a predictor of future decay (Jordan, Laraway et al., 1987; Hildebrandt and Bretz, 2006; Thaweboon, Thaweboon et al., 2006; Sanchez-Garcia, Gutierrez-Venegas et al., 2008). These tests mainly focus on the identification of mutans streptococci or lactobacilli, as these groups of bacteria are considered the main offenders in
the development of dental caries (Bowden, 1997). However, due to the likely variance in virulence between strains within an individual bacterial species, bacterial counts per se do not necessarily correlate with caries risk (Lindquist and Emilson, 2004). The aciduricity and acidogenicity of the micro-organisms present are key factors in caries risk.

Other tests have focused on measuring the acid produced by cariogenic bacteria, rather than focusing on the individual bacterial species (Snyder, 1940; Rickles, 1953; Bretz, Corby et al., 2007; Chaussain, Opsahl, Vital et al., 2010). These tests have the benefit of measuring the effectors of demineralisation, organic acids, which are the end products of carbohydrate metabolism. These tests, however, often take up to days to obtain a result, and frequently over-predict patients at a high risk of developing caries (Chaussain, Opsahl, Vital et al., 2010).

The aim of this study was to evaluate a technique to predict white spot lesion development in patients undergoing fixed appliance orthodontic treatment. The hypothesis was that the acid producing capability of salivary microflora in an acidic environment, is a sufficiently accurate way to predict the risk of enamel demineralisation occurring in orthodontic patients undergoing fixed appliance treatment.
Materials and Methods

Approval for this study was granted through the University of Adelaide and the Adelaide Dental Hospital committees for the ethical use of humans in clinical trials. A power study was completed and indicated a minimum of fifty patients were necessary to provide statistically significant results. To allow for patients who might leave the study before completion, sixty patients from the Adelaide Dental Hospital Orthodontic Unit, who were to undergo fixed appliance treatment, were asked to participate in the study. The inclusion criteria included:

1. The treatment plan was to be full-arch fixed appliances,
2. Patients had not commenced treatment,
3. Patients were to be free from active caries or demineralisation as determined by both radiographic and clinical examination,
4. The oral hygiene level was deemed to be of a high standard with tooth surfaces free from plaque deposits,
5. Participation was voluntary and no variation to routine treatment and oral hygiene management was undertaken.

Eight patients failed to proceed with treatment, leaving fifty-two subjects in the study. Each patient was assigned a unique number for the study such that the examiner was blinded to the subject’s identity during testing and analysis.

A saliva sample was collected in a sterile tube prior to placement of appliances and at each subsequent routine orthodontic appointment, for the duration of the study. A minimum of 3 samples were collected from each subject (range 3 to 6), over an average time of 15.7 weeks (range 6 to 38 weeks). Patients were asked not to eat, drink or brush their teeth for at least 30 minutes prior to saliva collection.
Using two clean collection tubes per subject, 0.5ml of saliva was pipette into each tube and mixed with 0.5ml of a 0.4M potassium phosphate buffer pH 5.7 solution, either with or without the addition of 10% sucrose (w/v). The sample without sucrose acted as the negative control. The pH of both control and test samples was determined at 0 minutes using a calibrated Cyberscan 510pH meter with a Ross semi-micro combination pH electrode (Thermo Scientific, USA). After 30 minutes at 37°C in a water bath, the pH was again recorded and the difference between 0 and 30 minutes was calculated for each sample. This value was taken as the amount of change due to acid production by the salivary microflora.

At the completion of the time period available for the study, pre-treatment intra-oral photographs were compared to standardized intra-oral photographs taken at the last collection appointment, for the presence of white spot lesions. All photographs were taken on an EOS 400D Canon digital SLR 10 Mega Pixel camera with a EF100mm F2.8 macro-lens and MR-14EX ring flash under the same lighting conditions and magnification.

The photographs were re-examined by the same examiner 4 weeks after the initial comparison, as well as by an independent examiner with knowledge of white spot lesion development in orthodontic patients. The examiners were blinded to the subjects’ identity when examining for white spot lesions and teeth anterior to, and including, the first molars were examined. A subject was given a yes/no score to the presence of any white spot lesions.

Intra- and inter-examiner agreement was assessed using Kappa statistics. Results indicated a very good intra-examiner rating (1.00) and a good inter-examiner rating (0.73).
Results

All statistics were performed by an independent statistician using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA).

At the completion of the study, three subjects (5.8%) were identified who had developed at least one white spot lesion during the course of their orthodontic treatment. Lesions developed on the cervical half of the smooth buccal surfaces. Severity was not analysed as the presence of a white spot lesion anywhere is indicative of the requirement of preventive treatment.

After analysis of the results, a decrease in pH of 0.15 units was arbitrarily chosen as the delineator between high and low risk subjects. Five of the fifty-two subjects were deemed high risk, while the remaining forty-seven were placed into the low-risk group. Two of the patients who developed white spot lesions were found to be in the high risk group, while one was rated low-risk.

Initial pH change, assigned risk category and white spot lesion development, are shown in table 1. Those who developed white spot lesions are highlighted.

Of the three participants who developed white spot lesions, two were categorised as positive by the test (67% sensitivity). Of the 49 participants who did not develop white spot lesions, 47 were categorised as low risk by the test (94% specificity). Of the five participants who were rated high risk by the test, two went on to develop white spot lesions (40% positive predictive value). Of the 47 participants who were rated negative by the test, 46 did not develop white spot lesions (98% negative predictive value).

To determine the association between the diagnostic test and white spot lesion outcome, a Fisher's exact test was performed and statistical significance
(p=0.021) was found, suggesting that the dichotomised version of baseline change in pH was a significant predictor of white spot lesion at final follow-up.

<table>
<thead>
<tr>
<th>Initial pH change (-ve denoted a reduction in pH)</th>
<th>Risk Category</th>
<th>White spot lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.27</td>
<td>high</td>
<td>no</td>
</tr>
<tr>
<td>-0.18</td>
<td>high</td>
<td>no</td>
</tr>
<tr>
<td><strong>-0.16</strong></td>
<td><strong>high</strong></td>
<td><strong>yes</strong></td>
</tr>
<tr>
<td>-0.16</td>
<td>high</td>
<td>no</td>
</tr>
<tr>
<td><strong>-0.15</strong></td>
<td><strong>high</strong></td>
<td><strong>yes</strong></td>
</tr>
<tr>
<td>-0.13</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.09</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.06</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.06</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.05</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.04</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.03</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.03</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.02</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.02</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.02</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.02</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.02</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.02</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.02</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.02</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.01</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.01</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>-0.01</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td><strong>0</strong></td>
<td><strong>low</strong></td>
<td><strong>yes</strong></td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
<tr>
<td>0</td>
<td>low</td>
<td>no</td>
</tr>
</tbody>
</table>

Table 1. Initial pH change, risk category, and white spot lesion development in patients undergoing fixed orthodontic treatment.
<table>
<thead>
<tr>
<th>Initial collection</th>
<th>Day of Banding</th>
<th>Collection 3</th>
<th>Collection 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>-0.07</td>
<td>-0.09</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-0.02</td>
<td>0</td>
</tr>
<tr>
<td>-0.03</td>
<td>-0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-0.09</td>
<td>-0.01</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-0.03</td>
<td>0</td>
</tr>
<tr>
<td>-0.02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.16</td>
<td>-0.01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>-0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.02</td>
<td>-0.02</td>
<td>0</td>
<td>-0.04</td>
</tr>
<tr>
<td>-0.15</td>
<td>-0.02</td>
<td>0</td>
<td>-0.02</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-0.04</td>
</tr>
<tr>
<td>-0.06</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.06</td>
<td>-0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.16</td>
<td>-0.07</td>
<td>-0.02</td>
<td>-0.05</td>
</tr>
<tr>
<td>0</td>
<td>-0.02</td>
<td>-0.06</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>-0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>-0.01</td>
<td>-0.01</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-0.03</td>
</tr>
<tr>
<td>-0.18</td>
<td>-0.02</td>
<td>-0.09</td>
<td>0</td>
</tr>
<tr>
<td>-0.04</td>
<td>0</td>
<td>-0.07</td>
<td>0</td>
</tr>
<tr>
<td>-0.02</td>
<td>0</td>
<td>-0.02</td>
<td>-0.02</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-0.13</td>
</tr>
<tr>
<td>-0.02</td>
<td>0</td>
<td>0</td>
<td>-0.01</td>
</tr>
<tr>
<td>0</td>
<td>-0.01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.13</td>
<td>-0.07</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.02</td>
<td>0</td>
<td>-0.05</td>
<td>-0.02</td>
</tr>
<tr>
<td>-0.02</td>
<td>0</td>
<td>0</td>
<td>-0.03</td>
</tr>
<tr>
<td>-0.01</td>
<td>0</td>
<td>-0.24</td>
<td>0</td>
</tr>
<tr>
<td>-0.03</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.02</td>
<td>0</td>
<td>-0.01</td>
<td>0</td>
</tr>
<tr>
<td>-0.03</td>
<td>0</td>
<td>-0.10</td>
<td>0</td>
</tr>
<tr>
<td>-0.09</td>
<td>0</td>
<td>0</td>
<td>-0.01</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.09</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.27</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>-0.04</td>
<td>0</td>
<td>-0.01</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-0.01</td>
</tr>
</tbody>
</table>

Table 2. Change in salivary pH for each subject over time.

Table 2 shows the pH change for each subject over time. There is a time interval of six to eight weeks between each collection. Difference in the number
of samples collected for each subject was affected by attendance of appointments and length of time in the study.

Several variations over time among individual subjects were seen in pH change. A linear mixed effects model was fitted to the data. These changes were not statistically significant ($P=0.38$) indicating a patients’ caries risk did not tend to change during treatment.
Discussion

With a reported incidence of white spot lesion development in patients undergoing fixed orthodontic treatment being as high as 50% (Gorelick, Geiger et al., 1982; Lundström and Krasse, 1987; Lovrov, Hertrich et al., 2007), a test to identify those patients at a higher risk before the caries process begins would be useful in directing resources aimed at preventing enamel mineral loss. The majority of chair-side saliva tests currently available determine bacterial numbers, specifically mutans streptococci and lactobacilli. Currently, there is no test to suit all individuals that reliably identifies at-risk patients (Hausen, 1997; Reich, Lussi et al., 1999; Zimmer, Bizhang et al., 2008). One reason may be that other cariogenic species are not commonly targeted in saliva tests, e.g. Actinomyces species, which contribute to the aciduric microflora in the oral cavity. These bacteria might contribute to an individual’s development of enamel demineralisation (Sansone, van Houte et al., 1993; Beighton, 2005).

The aim of the present study was to measure the end product of refined carbohydrate metabolism which leads to mineral loss; specifically, acid production of all salivary bacteria present.

From the results, it is possible that this technique has the potential to be used as a commercial chair-side saliva test indicating whether the patient is at an increased risk of developing white spot lesions during their orthodontic treatment. It has been reported that a sensitivity in the range of 0.60-0.75 with specificity values about 0.80 may be adequate to ensure reasonable ‘case finding’ in terms of high-increment children (Bader, Graves et al., 1986). Kingman et al. (1990) reported that the sum of sensitivity and specificity should be at least 160% before a caries risk marker can be considered a legitimate
candidate for targeting individualized prevention. With sensitivity of 67% and specificity of 94%, and a combined score of 161%, this test appears to satisfy both of the previously reported papers’ requirement for a legitimate caries risk test.

The results indicate that this test more accurately predicts those patients who fall into the low risk category, and tends to over-predict those at an increased risk of developing white spot lesions. This is reported in the literature as being the case for most commercial saliva tests (Hausen, 1997). While this certainly is not as ideal as having a test with 100% specificity and sensitivity, the current test is simple and has the ability to reduce the number of potential patients who would require additional preventive treatment to avert demineralisation during treatment, thus utilising resources more effectively. In addition, the test could assist the practitioner to identify patients who may benefit from preventive measures to reduce the numbers of potentially cariogenic bacteria prior to starting treatment, in an attempt to reduce their risk of white spot lesion development. Overall, the published data tend to indicate that the prediction of high caries risk children, on the basis of a single microbial factor, is difficult whereas the prediction of low caries risk is more reliable (Matsumoto, Sugihara et al., 2006; Chaussain, Opsahl Vital et al., 2010).

One limitation of this study was that due to time constraints of the ethics approval for the clinical trial, the patients were unable to be followed over the entire course of their treatment. A more comprehensive analysis of white spot lesion development would be gained by assessing the patients following the removal of appliances, with a careful prophylaxis and thorough clinical examination. While assessing the lesion development with intra-oral
photographs had a good intra- and inter-examiner reliability, 1.00 and 0.73 Kappa scores respectively, it is possible that more patients may have developed detectable demineralisation during treatment, with potential to affect the test sensitivity and specificity. Continuing research aims to follow the patient cohort over the entirety of their treatment.

The purpose of taking unstimulated saliva in this test was to analyse the potential for acid formation in the absence of the buffering capacity. Stimulated saliva contains various components designed to neutralise the pH rapidly following an acid attack. In resting saliva, the major buffering agent is inorganic phosphate (Tenovuo, 1997). The potassium phosphate solution used in this test was designed to saturate any buffering capacity of the saliva present, ensuring the environment was controlled and only the single variable of pH change was being measured.

Occasionally, patients exhibited variation in salivary acid production rates over time. This is most likely due to changes in oral hygiene after appliances have been placed. In cases where acid production increased, a likely explanation is that oral hygiene has not been maintained to pre-treatment standards; leading to higher levels of cariogenic bacteria. This has been reported in numerous studies (Owen, 1949; Bloom and Brown, 1964; Demling, Heuer et al., 2009).

Several patients who started with a high rate of acid production improved over time, potentially reducing their caries risk. This has been reported in the literature by Southard and Cohen et al. (1986) who found that orthodontically treated patients had better dental health, in respect to caries development, than non-orthodontically treated patients.
Conclusion

This simple saliva test to identify a patient’s caries risk showed a sensitivity of 67% and specificity of 94% in the sample of fifty-two patients undergoing fixed appliance orthodontic treatment. It was more accurate at predicting patients who would not develop demineralisation during treatment than those who would. The test also confirmed there was no statistically significant change in the patient’s risk status during treatment.

This technique has the potential to be developed into a commercial chair-side saliva test, which produces results in 30 minutes, is simple, inexpensive and would identify those patients who may benefit from additional preventive precautions prior to and during orthodontic treatment.
References


Article 2

This article has been written in the format for submission to the Journal of Dental Research.
Salivary analysis of patients undergoing fixed orthodontic treatment; characterization of cariogenic bacterial strains

(Journal of Dental Research)

Dr. Sara Roberts
School of Dentistry
The University of Adelaide
Adelaide
South Australia
5005

Professor Wayne Sampson
Begg Chair in Orthodontics
The University of Adelaide
Adelaide
South Australia
5005

Dr. Neville Gully
School of Dentistry
The University of Adelaide
Adelaide
South Australia
5005

Assoc. Professor Craig Dreyer
School of Dentistry
The University of Adelaide
Adelaide
South Australia
5005
Salivary analysis of patients undergoing fixed orthodontic treatment; characterization of cariogenic bacterial strains
Abstract

Introduction: Specific virulence factors of cariogenic bacteria influence an individual’s risk of developing dental caries (Kohler and Krasse, 1990; Macpherson, MacFarlane et al., 1992). The aim of this study was to isolate strains of mutans streptococci from patients undergoing orthodontic treatment and identify variations in their ability to metabolise sucrose. Materials and Method: Twenty patients were selected who had been identified as high or low rate acid producers from a previous study. Saliva samples from patients were cultured onto selective agar plates and mutans streptococci colonies identified and enumerated. Purified MS strains from each were cultured in tryptone-soya broth containing sucrose and following 48 hours of incubation at 37°C, the growth, terminal pH and production of organic acids were recorded and analysed. Results: There was significant variation in metabolism among individual strains, however this did not correlated with high or low acid production. Conclusion: Individual MS strains showed a high variation in the metabolism of sucrose.
Introduction

Full fixed orthodontic appliance treatment poses significant risks to both the hard and soft tissues. One of the most common complications is the demineralization and subsequent white spot lesion development in the enamel (Travess, Roberts-Harry et al., 2004). White spot lesions are the early indication of dental caries. The incidence of white spot lesions in orthodontic patients has been reported as being as high as 50 per cent (Gorelick, Geiger et al., 1982; Lundström and Krasse, 1987; Lovrov, Hertrich et al., 2007) sometimes occurring as early as one month after the placement of the appliances (Ogaard, Rølla et al., 1988).

It is thought that the placement of a full fixed appliance increases the difficulty of mechanical removal of plaque. Orthodontic bands and brackets are thought to influence the microflora of the mouth (Sakamaki and Bahn, 1968; Demling, Heuer et al., 2009), and increases in species of lactobacilli, streptococci, staphylococci, and actinomyces species have been reported (Owen, 1949; Bloom and Brown, 1964; Kim, 2009; Costa, 2010). While patient plaque scores do not seem to be an indicator of risk (Al Mulla, Kharsa et al., 2009), historically the numbers of mutans streptococci and lactobacilli in saliva have been used as an indicator of caries risk. This confirmed that the quantity of specific cariogenic microorganisms present in the patient’s oral cavity, rather than the plaque mass, is responsible for enamel demineralisation.

It has been reported that bacterial strains within a species can show a wide variation of characteristics (Davey and Rogers, 1984; de Soet, van Loveren et al., 1991; Macpherson, MacFarlane et al., 1992; Bowden, 1997). The loss or acquisition of virulence factors by a particular strain of cariogenic bacteria
could, therefore, influence the caries experience of the individual. One of the most important aspects in the identification of variants of pathogenic bacteria is that, in some individuals, this can be associated with virulence and, therefore, possibly caries risk. It has been reported that the existence of many variants of a species is typical of both commensal organisms and/or opportunistic pathogens (Loos, Dyer et al., 1993). In the resident oral flora of humans, several subgroups of the same species may exist within one environment (Bowden, 1997). It may be possible that specific species subgroups have unique functions within the oral environment. The streptococci are the major acid producers in dental plaque (Iwami, Hata et al., 1989) and Streptococcus mutans metabolises a wider variety of carbohydrates than any of the Gram +ve micro-organisms indentified so far (Mitchell, 2003). The glycolytic pathway leads to the production of pyruvate, which in turn can be metabolised to a mixture of other acids (Mitchell, 2003); however under conditions of excess fermentable carbohydrate, lactic acid is almost exclusively produced (Duguid, 1985).

The aim of this study was to compare the mutans streptococci strains isolated from high and low caries risk groups of patients undergoing orthodontic treatment in order to identify any differences in bacterial metabolism of sucrose between them.
Method and Materials

Approval for this study was granted through the University of Adelaide and the Adelaide Dental Hospital committees for the ethical use of humans in clinical trials. Twenty patients were identified for this study from an earlier investigation of caries risk of orthodontic patients. Two groups of ten patients were identified as having either low or high caries risk, on the basis of the combined acidogenicity and acidoduricity of their salivary microflora. These patients were all undergoing fixed appliance orthodontic treatment in the Adelaide Dental Hospital Orthodontic Unit.

A sample of saliva was collected from each patient and placed into a sterile tube. Each saliva sample was serially diluted 10-fold three times in sterile saline solution (0.9% w/v). Duplicate 25 µL aliquots of each dilution were inoculated onto TSY20B agar plates. TSY20B is a selective medium for mutans streptococci and contains tryptone soya broth, yeast extract, sucrose (20% w/v) and bacitracin (appendix 1). Bacitracin is an antibiotic which is selective for mutans streptococci among other bacteria. The plates were incubated for 48 hours at 37°C and mutans streptococci colonies were counted with the aid of a dissecting light microscope. Once identified, mutans streptococci colonies were transferred onto to a fresh TSY20B plate in order to purify strains from each saliva sample. Two of the strains from the high risk group could not be isolated and cultured, therefore leaving eighteen strains for comparison.

Pure bacterial samples were seeded into 9ml sterile tryptone soya (TS) broth containing 10% sucrose (w/v) and incubated for 48 hours. Following growth in TS sucrose broth, the pH of each sample was measured using a calibrated
Cyberscan 510pH meter with a Ross semi-micro combination pH electrode (Thermo Scientific, USA). In addition, the optical density of each broth was measured at a wavelength 560 nm using a dual beam Perkin-Elmer, Lambda 5, UV/VIS Spectrophotometer, using sterile broth as reference.

In order to analyse acidic end-products of fermentation, 1ml of the culture was centrifuged at 4000RPM for 4 minutes at 4°C in a IEC/Centra ® MP4R centrifuge. Following centrifugation, the resultant supernatant was removed from the cell pellet by pipette and organic acid analysis conducted using ion exclusion high pressure liquid chromatography (see appendix 2) This method is based on that described by (Guerrant, Lambert et al., 1982). Known concentrations of lactic, acetic and formic acids, were used as external standards. Unknown samples were analysed by HPLC to identify and quantify organic acids arising from the fermentation of sucrose by the bacteria. The quantities for each acid were determined by comparison of peak retention time and area to the known standard for each organic acid.
Results

All statistical calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA) by an independent statistician.

Comparison among populations

The results are displayed in Tables 1 and 2 for the high and low risk groups respectively.

From the high risk group, five of the subjects (62.5%) possessed numbers of salivary mutans streptococci above $10^6$ cfu/ml, a value reported in the literature as being associated with an increased risk of developing dental caries (Kingman, 1988; Russell, MacFarlane et al., 1991; Alaluusua, 1993). In comparison, the low risk group contained three of the ten subjects (30%) with bacterial numbers exceeding this level.

Results for growth characteristics for each of the purified mutans streptococci isolates from each subject are also found in Tables 1 and 2.

A Wilcoxon test was applied to the data in order to compare differences between the two groups as a population. The Wilcoxon test is the non-parametric equivalent of the t-test. This showed that when comparing the two groups, none of the results were statistically significant ($p > 0.05$).
Table 1. Results for high risk group, showing MS CFU/ml, cell yield of culture, terminal pH of broth, production of lactic and acetic acid from each strain of MS. Samples nine and ten have been excluded as pure strains could not be cultured.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Salivary MS Count</th>
<th>Cell yield (OD560)</th>
<th>Terminal pH</th>
<th>Lactic acid production (mM/% of total*)</th>
<th>Acetic acid production (mM/% of total*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 x 10^5</td>
<td>0.47</td>
<td>5.94</td>
<td>12.3/58</td>
<td>8.8/42</td>
</tr>
<tr>
<td>2</td>
<td>6 x 10^5</td>
<td>1.27</td>
<td>3.99</td>
<td>48/86</td>
<td>7.5/14</td>
</tr>
<tr>
<td>3</td>
<td>4 x 10^6</td>
<td>1.45</td>
<td>4.06</td>
<td>44.1/82</td>
<td>10/18</td>
</tr>
<tr>
<td>4</td>
<td>2.4 x 10^5</td>
<td>0.64</td>
<td>5.74</td>
<td>16.9/66</td>
<td>8.8/34</td>
</tr>
<tr>
<td>5</td>
<td>2 x 10^6</td>
<td>0.54</td>
<td>5.96</td>
<td>14/65</td>
<td>7.5/35</td>
</tr>
<tr>
<td>6</td>
<td>1.6 x 10^6</td>
<td>0.66</td>
<td>5.88</td>
<td>16.2/68</td>
<td>7.5/32</td>
</tr>
<tr>
<td>7</td>
<td>1.6 x 10^6</td>
<td>0.54</td>
<td>3.97</td>
<td>48.5/87</td>
<td>7.5/13</td>
</tr>
<tr>
<td>8</td>
<td>1.2 x 10^7</td>
<td>1.01</td>
<td>4.49</td>
<td>33.3/82</td>
<td>7.5/18</td>
</tr>
</tbody>
</table>

Table 2. Results for low risk group, showing MS CFU/ml, cell yield of culture, terminal pH of broth, production of lactic and acetic acid from each strain of MS.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Salivary Count</th>
<th>Cell Yield (OD560)</th>
<th>Terminal pH</th>
<th>Lactic acid production (mM/% of total*)</th>
<th>Acetic acid production (mM/% of total*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 x 10^5</td>
<td>0.88</td>
<td>5.27</td>
<td>18.6 / 71</td>
<td>7.5 / 29</td>
</tr>
<tr>
<td>2</td>
<td>1.6 x 10^5</td>
<td>0.61</td>
<td>5.45</td>
<td>17.6 / 72</td>
<td>6.9 / 28</td>
</tr>
<tr>
<td>3</td>
<td>2 x 10^5</td>
<td>0.42</td>
<td>6.32</td>
<td>7.8 / 53</td>
<td>6.9 / 47</td>
</tr>
<tr>
<td>4</td>
<td>4 x 10^5</td>
<td>0.7</td>
<td>3.98</td>
<td>50.9 / 87</td>
<td>7.5 / 13</td>
</tr>
<tr>
<td>5</td>
<td>2.8 x 10^5</td>
<td>2.06</td>
<td>3.87</td>
<td>53.4 / 88</td>
<td>7.5 / 12</td>
</tr>
<tr>
<td>6</td>
<td>8 x 10^5</td>
<td>0.69</td>
<td>4.08</td>
<td>43.1 / 84</td>
<td>8.1 / 16</td>
</tr>
<tr>
<td>7</td>
<td>1.6 x 10^5</td>
<td>0.16</td>
<td>5.81</td>
<td>15.2 / 63</td>
<td>8.8 / 37</td>
</tr>
<tr>
<td>8</td>
<td>6 x 10^5</td>
<td>1.73</td>
<td>3.94</td>
<td>50.9 / 87</td>
<td>7.5 / 13</td>
</tr>
<tr>
<td>9</td>
<td>3.2 x 10^5</td>
<td>1.65</td>
<td>4</td>
<td>44.6 / 84</td>
<td>8.8 / 16</td>
</tr>
<tr>
<td>10</td>
<td>2 x 10^6</td>
<td>1.46</td>
<td>3.85</td>
<td>54.4 / 88</td>
<td>7.5 / 12</td>
</tr>
</tbody>
</table>

*Total acid production refers to the combined lactic and acetic acid concentrations

**Association between, salivary mutans levels, biomass, terminal pH and organic acid production**

Optical densities, a measure of cell biomass, in pure cultures of mutans streptococci varied between 0.16 to 2.06 absorbance units (AU). Higher optical densities indicate the bacteria were able to grow at a faster rate. Terminal pH in cultures of pure stains from individuals also varied between 3.85 and 5.96. It appeared that those strains that produced greatest biomass had the ability to produce more acid as the terminal pH in these cultures tended to be lowest.
Lactic acid was the dominant organic acid produced by each of the purified mutans streptococci strains, with production of acetic acid also detected at lower concentrations.

The association between the measures was assessed using Spearman correlation coefficients.

Total bacterial counts were not statistically associated with any of the measures.

Optical density was statistically associated with terminal pH ($P=0.0002$) and lactic acid production ($P<.0001$).

Terminal pH was statistically associated with optical density ($P=0.0002$) and lactic acid production ($P<.0001$).
Discussion

The development of dental caries is a complex, multifactorial process. It has been concluded that there is not one single factor explaining the changes observed. Thus, in one population, different explanations may be relevant for different individuals, for different age groups, for different teeth and for different periods of time (Bratthall, 1996). The presence of cariogenic bacteria is, however, an integral part in this process (Bowden, 1997). There is sufficient evidence in the literature to indicate that there are variables within a species of bacteria, which may influence that individual’s risk of developing the disease (Kohler and Krasse, 1990; Macpherson, MacFarlane et al., 1992).

High levels of mutans streptococci in saliva and plaque are associated with a high caries incidence. They are both acidogenic and aciduric (Denepitiya and Kleinberg, 1984). Streptococci are the major acid producers in dental plaque (Iwami, Hata et al., 1989). *Streptococcus mutans* can metabolise a wider variety of carbohydrates than any other Gram +ve micro-organisms indentified so far, and genes for the transport and metabolism for these carbohydrates are present in its genome (Mitchell, 2003).

Most traditional tests employed for predicting caries risk involve the sampling of saliva and subsequent culturing and enumeration of cariogenic bacteria. In patients, levels at or above $10^6$ cfu/ml of mutans streptococci in saliva has been historically used as an indicator of increased risk of developing dental caries (Kingman, 1988; Russell, MacFarlane et al., 1991; Alaluusua, 1993). As virulence is a variable amongst pathogens, perhaps the ability to identify highly pathogenic strains within cariogenic species of bacteria present in an individual’s oral cavity might lead to a more accurate prediction of caries risk.
This study looked at a small population of patients undergoing fixed appliance orthodontic treatment to identify differences in the metabolism of mutans streptococci strains isolated from the group.

The twenty subjects were selected from a larger cohort of subjects who had volunteered for an analysis of a modified saliva test with the potential to predict dental caries. The change in pH of a subject’s saliva, following sucrose challenge over 30 minutes, was used to determine their risk of developing dental caries. Therefore, risk was assigned according to the ability of cariogenic bacteria in saliva to metabolise and produce acid. It was thought that bacterial species that could produce acid most rapidly would be more likely to contribute to the caries process. Therefore, ten patients who exhibited the highest rates of acid production and ten who had no or minimal change in salivary pH were selected for further analysis of their salivary bacteria. Ideally, more patients would have made the testing more powerful; however, the study was limited by the number of patients exhibiting large salivary pH changes.

While the results comparing the two groups were not statistically significant, interestingly, the high risk group showed a higher percentage of patients with salivary MS greater than $10^6$ cfu/ml. An increased number of subjects in the study may have shown significant results.

As expected, the most common acid produced by the mutans streptococci in each subject was lactic acid. This has been confirmed by many studies in the past (Abbe, Takahashi et al., 1982; Iwami, Hata et al., 1989; Jacobson, Lodge et al., 1989). While it has been reported that several organic acids can be the end result of metabolism of fermentable carbohydrates, including lactic, acetic,
formic, propionic and succinic acids (Geddes, 1975), in this study, only lactic and acetic acids were detected. In the presence of excess fermentable carbohydrates, lactic acid is rapidly produced almost exclusively (Minah and Loesche, 1977; Duguid, 1985), and this was confirmed by these results.

**Limitations**

There were several limitations in this study. As previously mentioned, a larger sample size would have been preferable, however due to only 10 subjects meeting the criteria for high acid production, we were limited to matching 10 low acid producing subjects, leaving only 20 in the study. A larger sample size may have produced more significant results.

We were unable to isolate pure mutans streptococci strains from 2 of the high risk subjects due to mould spore contamination on the agar plates. This left an even smaller number in the high risk group. This could be prevented in the future by initially plating 2 TSY20B agar plates per subject.

It would be recommended that future research in this area focus on larger sample sizes, as well as looking at subjects with higher levels of acid production than those seen in this study. This has the potential to provide further insight into the metabolic differences between individual strains of mutans streptococci in relation to caries risk.
Conclusion

Differences between the sucrose metabolism of mutans streptococci strains did not appear to have a statistically significant correlation to the rate of acid production of the total oral salivary microflora among the two populations. Within the individual strains, there were significant associations between cell yield, terminal pH and lactic acid production, with results indicating that a higher cell yield was associated with a lower terminal pH and higher levels of lactic acid production.

Between the populations, there was a large range in each of the metabolism characteristics measured, indicating that while there may not be significant differences between population groups, there was a range of strains present, with varying virulence.

This study confirms that caries risk cannot be assessed by looking at one species of cariogenic bacteria only, rather the cariogenicity of the total oral microflora should be taken into consideration when assessing a patient’s risk status.
References


Summary

The first part of this research analysed a simple saliva test using acid production of the salivary microflora as a whole as an indicator of caries risk in the short term in patients undergoing fixed orthodontic treatment. Results indicated that both the specificity and sensitivity satisfy the accuracy reported in the literature as being required for caries prediction (Bader, Graves et al., 1986, Kingman et al., 1990).

There were several limitations with this research. Sixty participants gave consent to be part of this study, however, eight did not proceed with orthodontic treatment, therefore were not included in the results. This left only fifty-two participating in the research. While a power study indicated that a minimum of fifty subjects would be required to achieve statistically significant results, perhaps a larger cohort would have provided more significant results. Only three of the fifty-two subjects developed identifiable white spot lesions.

A second limitation was that due to time constraints of the ethics approval for the clinical trial, the patients were unable to be followed over the entire course of their treatment. A more comprehensive analysis of white spot lesion development would be gained by assessing the patients following the removal of appliances, with a prophylaxis and thorough clinical examination. It is recommended that this cohort of patients be followed through to the completion of their orthodontic treatment for further analysis of white spot lesion development.

This test appears to predict low risk patients more accurately than high risk patients. This has been reported as being the case for most caries predictive tests (Hausen, 1997). Further analysis of these subjects at the completion of
orthodontic treatment may change the sensitivity and specificity of this test, thus changing the overall accuracy of prediction.

It is recommended that this test be used as a screening tool to identify those patients who may benefit from additional preventive treatment, rather than a specific caries diagnostic test.

The second part to this research used a small cohort of subjects identified as either high or low caries risk from the initial test. Mutans streptococci strains were identified and analysed for differences in bacterial metabolism of sucrose. Differences in the metabolism of individual's strains did not appear to have a statistically significant correlation to the rate of acid production of the total oral salivary microflora among the two populations.

The small sample size was a limitation; however, due to the small number of subjects classified as high risk according to the acid production of salivary microflora, we were limited to only ten high and ten low risk individuals. It would be recommended that future research in this area include larger sample sizes, as well as looking at subjects with higher levels of acid production than those seen in this study. This has the potential to provide further insight into the metabolic differences between individual strains of mutans streptococci in relation to caries risk.
APPENDIX 1

Materials

TSY20B agar plates

Preparation
15g tryptone soya broth powder
7g yeast extract (1-2% w/v)
100g sucrose (20% w/v)
7.5g agar (1.5% w/v)
500ml de-ionised water
1ml bacitracin

Procedure
Mix all materials excluding the bacitracin and place in waterbath 110°C to dissolve sucrose. Autoclave solution to sterilize. Cool to 60°C then add bacitracin. Pour into sterile agar plates and leave to set. Seal in airtight packaging and keep in cool room.
Solutions

0.4M potassium phosphate buffer solution 5.7 pH-control

Preparation
27.2 g potassium di-hydrogen orthophosphate (KH₂PO₄) dissolved in 500ml de-ionised water (acidic solution)
34.8g di-potassium hydrogen orthophosphate (K₂HPO₄) dissolved in 500ml de-ionised water (basic solution)

Procedure
In a glass beaker, using a calibrated pH probe, mix the two solutions until a pH 5.7 is reached. Results from an unpublished pilot study testing this method, pH 5.7 was shown to be a delineator for the survival aciduric and non-aciduric cariogenic species.

0.4M potassium phosphate buffer solution 5.7 pH plus 10% sucrose-test

Preparation
Potassium phosphate buffer solution mixed with 10% sucrose (w/v)

Tryptone-Soya Broth

Preparation
15g tryptone soya broth powder dissolved in 500ml de-ionised water
10% sucrose (w/v)
APPENDIX 2

End-product acid analysis

Determination of acidic end-products was performed using ion-exclusion chromatography based on the method of Guerrant et al, 1982.

Instrumentation:
Rheodyne 7125 injector with 20 µl sample volume
Organic Acid Analysis HPLC Column (Aminex HPX-87H, Bio-Rad Laboratories)
Waters Model 501 HPLC Pump
Waters Model R401 Refractive Index Detector
Waters Model 730 Data Module
Waters Model 2259 Temperature Control Module

Mobile Phase:
This was an isocratic separation using 3.5mM H₂SO₄ at a flow rate of 1ml min⁻¹ for elution.
Cell-free culture filtrates were prepared and 100µl of these were injected for analysis. A standard solution containing the following compounds at a concentration of 10mM, acetate, lactate, formate, and glucose were used to determine peak retention times and area for quantitation.

Reference
Example of data module printout for HPLC analysis

Peak retention times

15.07 – lactic acid
17.92 – acetic acid
APPENDIX 3

Information sheet for participants

Information sheet for participants regarding the project titled “The use of saliva testing to predict the risk of decay development during orthodontic treatment”

The purpose of this study is to evaluate a test for the prediction of decay development during treatment with fixed braces. This research is going towards a thesis for a Doctor of Clinical Dentistry.

We will collect a sample of saliva at each of your orthodontic appointments. It will take approximately 5 minutes of your time each visit. This can be done before or after the orthodontist has seen you. At the end of the project you will be assessed for the development of any early decay. The collection of saliva will not affect your orthodontic treatment in any way.

If this test is successful it may be used as a quick way to determine if a patient is at a high risk of developing decay at any time during the course of treatment. The development of this test will not be completed during the course of your treatment so it will not be able to be used to assess your individual decay risk.

Your participation in this research project is strictly confidential and personal details will not be included.

You are able to withdraw from the study at any time without notice. This will not affect your orthodontic treatment in any way. If you have any complaints please see the attached independent complaints sheet.

Contact details

Research candidate
Dr Sara Roberts, ph. 83033102

Research Supervisors
Professor Wayne Sampson, ph. 83035153
Dr Craig Dreyer, ph. 83035153
Dr Neville Gully, ph. 83033887

School of Dentistry
Faculty of Health Sciences
The University of Adelaide

SA  5005
APPENDIX 4

Contact information for independent complaints procedure given to participants

THE UNIVERSITY OF ADELAIDE
HUMAN RESEARCH ETHICS COMMITTEE

Document for people who are participants in a research project

CONTACTS FOR INFORMATION ON PROJECT AND INDEPENDENT COMPLAINTS PROCEDURE

The Human Research Ethics Committee is obliged to monitor approved research projects. In conjunction with other forms of monitoring it is necessary to provide an independent and confidential reporting mechanism to assure quality assurance of the institutional ethics committee system. This is done by providing research participants with an additional avenue for raising concerns regarding the conduct of any research in which they are involved.

The following study has been reviewed and approved by the University of Adelaide Human Research Ethics Committee:

Project title: Evaluation of salivary flora acidogenicity under acidic conditions for prediction of cariogenic potential during fixed orthodontic treatment.

1. If you have questions or problems associated with the practical aspects of your participation in the project, or wish to raise a concern or complaint about the project, then you should consult the project co-ordinator:

   Name: Sara Roberts.
   telephone: (08) 83033102

2. If you wish to discuss with an independent person matters related to
   • making a complaint, or
   • raising concerns on the conduct of the project, or
   • the University policy on research involving human participants, or
   • your rights as a participant

   contact the Human Research Ethics Committee’s Secretary on phone (08) 8303 6028
APPENDIX 5

Consent forms for participants and guardians of a participant
THE UNIVERSITY OF ADELAIDE HUMAN RESEARCH ETHICS COMMITTEE

STANDARD CONSENT FORM
FOR PEOPLE WHO ARE PARTICIPANTS IN A RESEARCH PROJECT

1. I, ............................................................... (please print name)
   consent to take part in the research project entitled: “Evaluation of salivary flora acidogenicity under acidic conditions for prediction of cariogenic potential during fixed orthodontic treatment”

2. I acknowledge that I have read the attached Information Sheet entitled: “The use of saliva testing to predict the risk of decay development during orthodontic treatment”

3. I have had the project, so far as it affects me, fully explained to my satisfaction by the research worker. My consent is given freely.

4. Although I understand that the purpose of this research project is to improve the quality of orthodontic care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.

6. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.

7. I understand that I am free to withdraw from the project at any time and that this will not affect orthodontic advice in the management of my health, now or in the future.

8. I am aware that I should retain a copy of this Consent Form, when completed, and the attached Information Sheet.

................................................................. (signature) (date)

WITNESS

I have described to ................................................(name of participant)

the nature of the research to be carried out. In my opinion she/he understood the explanation.

Status in Project: .................................................................

Name: .................................................................

................................................................. (signature) (date)

THE UNIVERSITY OF ADELAIDE HUMAN RESEARCH ETHICS COMMITTEE
STANDARD CONSENT FORM
For Research to be Undertaken on a Child, and those in Dependant Relationships or Comparable Situations
To be Completed by Parent or Guardian

1. I, ____________________________________________________________ (please print name)
   consent to allow _______________________________________________ (please print name)
   to take part in the research project entitled: Evaluation of salivary flora acidogenicity under acidic conditions for prediction of cariogenic potential during fixed orthodontic treatment

2. I acknowledge that I have read the attached Information Sheet entitled: “The use of saliva testing to predict the risk of decay development during orthodontic treatment”

   and have had the project, as far as it affects __________________ (name)

   fully explained to me by the research worker. My consent is given freely.

   IN ADDITION, I ACKNOWLEDGE THE FOLLOWING ON BEHALF OF
   _________________________________________________________________(name)

3. Although I understand that the purpose of this research project is to improve the quality of orthodontic care, it has also been explained to me that involvement may not be of any benefit to him/her.

4. I have been given the opportunity to have a member of his/her family or friend present while the project was explained to me.

5. I have been informed that the information he/she provides will be kept confidential.

6. I understand that he/she is free to withdraw from the project at any time and that this will not affect orthodontic advice in the management of his/her health, now or in the future.

7. I am aware that I should retain a copy of this Consent Form, when completed, and the attached Information Sheet.

   ____________________________________________________________Parent/Guardian

   ____________________________________________________________

   (signature and please indicate relationship) (date)

WITNESS

I have described to ____________________________________________(name of parent/guardian)

the nature of the research to be carried out. In my opinion she/he understood the explanation.

Status in Project: ________________________________________________________________

Name: ________________________________________________________________

__________________________________________________________

(signature) (date)