Mutans Streptococci Genetic Strains in Children with Severe Early Childhood Caries: Implications for Caries Incidence and Treatment Outcome

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1 Introduction

1.1 Cariogenic Microorganisms and Severe Early Childhood Caries

Dental caries represents one of the most common chronic diseases affecting young children (Banas, 2004), and is a multifactorial disease involving complex interactions of genetic, dietary, environmental, behavioral, and microbial risk factors (Fejerskov, 2004). It is now accepted that dental caries is the result of changes in the plaque biofilm ecology brought about primarily by increased consumption of processed and simple carbohydrates. This leads to frequent and prolonged acidification of the plaque biofilm, resulting in selective pressures that lead to a more cariogenic oral microbiome dominated by acidophilic and aciduric microorganisms. Research over many decades strongly implicates several species of streptococci (especially mutans streptococci), lactobacilli, and actinomyces as among the most cariogenic of the well-characterized oral microbes (Kanasi et al., 2010). However over the last decade, DNA-based technologies have dramatically altered the view of the oral microbiome and expanded our understanding of the microbes associated with dental health and disease (Dewhirst, et al., 2010). This research has expanded the number of microorganisms associated with dental caries to include not only species of mutans streptococci, lactobacilli, and actinomyces, but also other species of non-mutans streptococci, bifidobacteria, and scardovia (Gross et al., 2012, Peterson et al., 2013). This has led to a refinement of the ecologic concept of caries (Marsh, 2003) to one defined by pathogenic communities (Jenkinson, 2011), similar to the current understanding of periodontal disease, another common plaque biofilm-associated pathology (Socransky & Haffajee, 2005).

Still it is widely acknowledged that S. mutans and S. sobrinus are among the most cariogenic microorganisms that reside in the oral cavity, and represent key bacteria associated with dental caries. There are distinct genotypes of S. mutans in saliva and dental plaque (Napimoga et al., 2005; Tabchoury et al., 2008), with some genotypes having preferred or dominant sites of localization in the oral environment (Napimoga et al., 2005; Tabchoury et al., 2008; Svensater et al., 1997). The coexistence and concurrent virulence of multiple mutans streptococci (MS) genotypes in caries-active individuals may serve as important determinants for increased caries incidence, as well as treatment success or failure (Svensater et al., 1997).

The development of dental caries requires consumed food substrates containing fermentable carbohydrates, susceptible tooth structures of the host, and the presence of cariogenic microorganisms (Zafar et al., 2009). Cariogenic microorganisms, including MS and lactobacilli, ferment the consumed carbohydrates and produce organic acids as by-products, which in turn demineralize tooth enamel hydroxyapatite and ultimately results in carious lesions (Zafar et al., 2009). Saliva is an important protective factor against caries development because it dilutes and buffers the acid produced by cariogenic microorganisms and provides calcium phosphate for remineralization. Saliva also contains IgA antibodies developed after the first exposure to the microorganisms (Cunha-Cruz et al., 2013).

The 1988-94 National Health and Nutrition Examination Survey (NIANES) found that 8.4 percent of 2-year old children had at least one decayed or filled tooth and that by age 5, 40.4 percent of the children were affected (Drury et al., 1999). Additionally, approximately 10.9% of children had frank caries and even higher percentages were found with enamel decalcifications or soft plaque accumulation (Yoon et al., 2012). Although there is a significant need for preventive and restorative treatment, only 11.7% of children in the United States received dental treatment in 2007 due to the limited access to dental care and the behavioral difficulties in restorative procedures for young children (Yoon et al., 2012).
S-ECC is defined in children ages 3-5 years old as: one or more cavitated, missing (due to caries) or smooth filled surfaces in primary maxillary anterior teeth, or dmfs (decayed, missing, filled and surface) score of >4 (age 3), >5 (age 4), or >6 (age 5) (Drury et al., 1999; Evans, 2013).

1.2 Mutans Streptococci and Virulence Factors for Dental Caries

*S. mutans* encode several potential virulence factors including biofilm formation, adhesion, acidogenesis, and acid tolerance. These would all be important factors when determining the epidemiology of dental caries. The ability of *S. mutans* to form biofilms on saliva-covered surfaces has been correlated with increased incidence of dental caries (Banas, 2004; Lemos et al., 2005; Nakano et al., 2002; Shemesh et al., 2007; Wen et al., 2004). Biofilm formation by *S. mutans* is essential for its ability to survive and thrive in a highly competitive environment. Two pathways of biofilm formation have been identified: sucrose-dependent and sucrose-independent adherence. The sucrose-dependent pathway is the more significant mechanism in the development of dental caries (Lemos et al., 2005). The major factors in the mechanism of sucrose-dependent adhesion are glucan-binding proteins (gbp) A, and C, and glucosyltransferase (gtf) B, C, and D, which promote adhesion (Lemos et al., 2005; Fujita et al., 2011; Bowen & Koo, 2011; Shemesh et al., 2007; Nakano et al., 2005). In the mouth, ingested dietary sucrose undergoes cleavage via gtf to glucose and fructose. Fructose is used as fuel by *S. mutans* whereas glucose is converted into dextran. These dextran chains allow for adhesion to enamel and constitute the scaffold in which *S. mutans* can colonize and form biofilm. Recently, the ComCDE operon, and the genetic regulators luxS, relA, and ccpA, have also been associated with biofilm formation (Lemos et al., 2005; Wen et al., 2004).

Adhesion of *S. mutans* is a major potential virulence factor of dental caries, and may also be involved in the development of infective endocarditis (Abranches et al., 2011). In the oral cavity, surface-associated protein P1 (spaP1) displays adherence to both collagen and enamel components (Lemos et al., 2005). Gbp and gtf also play significant roles in adhesion (Bowen et al., 2011; Fujita et al., 2011; Nakano et al., 2005; Shemesh et al., 2007). High levels of acid are major environmental stressors that affect the survivability of oral microorganisms (Banas, 2004; Guo et al., 2013; Lemos et al., 2005). Microorganisms that can survive at low pH would have distinct advantages in surviving in dental plaque (Wen & Burne, 2004; Matsui & Cvitkivitch, 2010; Lemos et al., 2005). The ability of *S. mutans* to tolerate acidic environments and compete with other bacterial flora is well documented and is a significant virulence factor. The major concern with acidic environments is the potential to acidify the intracellular cytoplasm. Detrimental effects of this acidification include DNA and protein damage, alteration of enzyme activity, and cell membrane damage. Acid tolerance mechanisms utilized by *S. mutans* include 1) an F-ATPase proton pump used to restore cytoplasmic pH, 2) DNA repair molecules uvrA and AP endonucleases, 3) use of cytoplasmic enzymes with a wide pH range such as LGL and pdhA, and 4) addition of membrane components to maintain physiological composition such as fabM, Dep, Ffh, and Dgk (Wen & Burne, 2004; Matsui & Cvitkivitch, 2010; Lemos et al., 2005; Cotter & Hill, 2003). The unique ability of *S. mutans* to maintain proper function under harsh acidic conditions has provided selective advantages for successful colonization of the oral dentition.

Acidogenesis is another important virulence factor that helps *S. mutans* compete in the oral cavity. *S. mutans* utilizes dietary sugars to produce and secrete lactic acid as an end-product of glycolysis (Palmier et al., 2012a; Banas, 2004; Dashper & Reynolds, 1996; Guo et al., 2013; Harris et al., 1992). As mentioned above, the acid tolerance of *S. mutans* is higher than in most bacteria; thus this microorganisms’ ability to acidify the environment eliminates or diminishes competing bacteria and allows for enhanced colonization (Wen & Burne, 2004; Lemos et al., 2005). More importantly, acid generation is the main
cause of tooth demineralization and tooth decay (Banas, 2004; Dashper & Reynolds, 1996; Guo et al., 2013; Harris et al., 1992). In addition to biofilm formation, dextran and other sugar polymer chains can serve as a carbon reservoir and ultimately increase the production of lactic acid (Lemos et al., 2005).

### 1.3 Prevention and Treatment of Dental Caries

Over the past few decades there has been considerable scientific progress that has greatly increased knowledge of the pathogenesis of dental caries. With these advances in scientific knowledge, combined with improved clinical techniques and dental materials, the clinical approach of treatment has evolved from an invasive surgical model to a minimal intervention medical model that attempts to address the cause of dental disease and not just the symptoms (Featherstone et al., 2012; Mount, 2007; Zero et al., 2001). These preventive treatment models involve assessing each individual’s risk for caries, or caries risk assessment (American Academy of Pediatric Dentistry Council on Clinical Affairs, 2013; Featherstone, 2003; Featherstone, 2004a, Litt et al., 1995; Nicolau et al., 2003). Once an individual’s risk has been assessed, a specific plan or “care path” can be implemented along with an adjunct customized restorative plan based on level of risk (American Academy of Pediatric Dentistry Council on Clinical Affairs, 2013; Ramos-Gomez & Ng, 2011). Multiple factors are taken into account when assigning caries risk status including socio-economic status, demographic background, attitude towards oral health, oral health habits, and systemic health and medications (American Academy of Pediatric Dentistry Council on Clinical Affairs, 2013; Featherstone, 2003, Featherstone, 2004b; Gao et al., 2013; Litt et al., 1995; Nicolau et al., 2003). Caries risk assessment has been shown to be an effective method of significantly reducing caries risk in individuals labeled as high and moderate risk (Featherstone et al., 2012).

Following the determination of caries risk status as low, moderate, or high, specific care paths can then be determined. This involves the use of minimally invasive restorative procedures based on evaluation of the extent of carious lesions as well as a customized oral hygiene regimen based on risk (Tassery et al., 2013). Two key tools that can be utilized to decrease or reverse caries progression are antibacterial agents and remineralizing agents (Featherstone & Doméjean, 2012). One of the most common agents utilized in caries prevention that works as both an antibacterial agent and remineralizing agent is fluoride (ten Cate & Featherstone, 2012). Fluoride has been widely known to prevent caries since the 1930s when naturally fluoridated water was shown to decrease caries prevalence in communities (Dean et al., 1938). It has since been demonstrated in numerous clinical trials that fluoride is an effective anticaries agent and is recognized as playing a central role in the decline of caries prevalence in many developed countries (Stookey, 1990; Hargreaves et al., 1983). Individuals of all different caries risk statuses will benefit from fluoride use, including use in dentifrices, topical applications, and mouthrinses (Zero, 2005). In addition to fluoride treatment, individuals at all caries risk levels may also benefit from a professional prophylactic cleaning and the use of a calcium phosphate-based paste (Tassery et al., 2013).

When discussing caries prevention, it is important to understand that caries is an infectious and transmissible disease. With this knowledge it is not surprising that colonization of cariogenic bacteria in the oral cavity of a child is generally the result of transmission from the child’s primary caregiver (Seki et al., 2006). In fact, a direct relationship can usually be found between levels of cariogenic bacteria in children and their primary caregivers (Douglass et al., 2008). It is now recognized that the implementation of an effecting perinatal program to improve a mother’s oral health may delay the acquisition of oral bacteria and the development of early childhood caries in their children (Ramos-Gomez, 2006). Following suit with the prevention and treatment techniques determined by caries risk assessment, it is critical that the parent/caregiver’s oral health be addressed when treating children (Ramos-Gomez et al., 2010). Such an
assessment includes the determination of biological and lifestyle risk factors that support the development and progression of caries (Ramos-Gomez et al., 2012).

1.4 Use of Xylitol in Prevention of Dental Caries

The use of xylitol as a preventive measure for dental caries has been controversial. This five-carbon sugar alcohol, which has been implemented as a food sweetener, is capable of reducing plaque formation, inhibiting enamel demineralization, and suppressing growth of plaque bacteria (Marsh et al., 2009). Xylitol, which can be delivered in numerous forms including lozenges, wipes, gum, and candy is widely considered to reduce caries by inhibiting the growth of MS (Marsh et al., 2009). It has been shown that consumption of xylitol can lead to a decrease in MS bound to dental plaque and a reduction in plaque acido-genicity (Marsh et al., 2009; Soderling, 2009). With these characteristics the use of xylitol as a preventive strategy for dental caries seems promising, however recent results from a placebo-controlled randomized trial of 691 participants suggests that the use of xylitol as an anticaries therapy for adults does not significantly reduce caries (Bader et al., 2013). This is contradicted by past studies where xylitol has been shown to significantly reduce caries incidence in young children (Milgram et al., 2012; Zhan et al., 2012). These contrasting results suggest the possibility that children may possess distinct MS strains with differing xylitol resistant properties.

The inhibitory effects of xylitol on MS may be credited to the inhibition of key bacterial glycolytic enzymes (e.g. phosphofructokinase). Xylitol is taken up by many strains of bacteria even if it cannot be metabolized (Waler & Rølla, 1990). This leads to the intracellular accumulation of xylitol 5-phosphate and the subsequent competition with bacterial glycolytic enzymes (e.g. phosphofructokinase), arrest of glycolysis, and impaired growth (Lee et al., 2012; ten Cate & Featherstone, 2012). Long-term xylitol consumption has been shown to cause the emergence of strains of xylitol-resistant MS. However, these xylitol resistant MS strains have been found to form biofilms of reduced depths and shed more easily from plaque into saliva (Trahan et al., 1992; Soderling et al., 1996). These less cariogenic traits result in reduced amounts of MS bacteria in plaque and potential decrease in transmission and colonization between individuals (Soderling et al., 1996; Palmer et al., 2012b).

The anticariogenic traits of xylitol may be compounded when taken in a chewing gum form. Chewing gum is known to increase salivary flow rates (Rebelles et al., 2010). Numerous oral health benefits can be credited to increased salivary flow. This includes more rapid oral clearance of sugars, neutralization of plaque acidic pH, and enhanced mineralization of early-carious lesions (Dodds, 2012). Due to these benefits, xylitol chewing gum may be a key tool in the prevention and treatment of dental caries.

1.5 Implications of Xylitol-Resistant Mutans Streptococci Strains in Children with Severe Early Childhood Caries

As the use of xylitol-containing products continues to gain popularity, questions emerge concerning the potential adaptation of plaque microorganisms, including MS, to xylitol and the possible selection of xylitol-resistant MS strains with increased cariogenic potential. Studies conducted in young children, as opposed to studies conducted with adults, indicate that xylitol (40% solution) may not significantly suppress S. mutans counts or plaque accumulation (Ramos-Gomez et al., 2012). Thus, caries-active children may potentially possess distinct MS genetic strains with differential xylitol resistance properties, with some strains also exhibiting increased cariogenic potential. One objective of this 1-year follow-up study was to provide insight on the use of xylitol treatment as an effective maintenance practice for caries preventive therapy in pediatric dental patients.
In this 1-year follow-up study, we examined the profiles of MS genotypic strains from a pediatric patient cohort that had been diagnosed with severe early childhood caries [S-ECC]. In the original study, isolates were collected both prior to and following full-mouth dental rehabilitation, which included the removal and/or repair of carious lesions and application of antimicrobial rinse and fluoride varnish. In this chapter, we examine the MS genetic strains that are dominant at 1-year post-dental rehabilitation and the emergence of six new previously undetected minor MS strains. We also characterize the xylitol resistance properties of select dominant and minor strains.

2 Methods

2.1 Patient Selection and Treatment

As described in our prior reports (Palmer et al., 2012a; Palmer et al., 2012b), participants for this study were selected from patients seen at the OHSU Pediatric Dentistry clinic. The inclusion parameters for recruitment were young children with S-ECC, but otherwise who had good general health. Exclusion criteria included children treated with antibiotics, topical fluoride application, and/or antiseptic mouth rinses within the previous three months, or undergoing orthodontic therapies. Study participants all underwent full-mouth dental rehabilitation therapy, conducted under general anesthesia at Doernbecher Children Hospital [located within OHSU], because this permitted the completion of full-mouth caries restorative therapy during a single patient visit. These individuals were all between 3-5 years of age.

The patient demographics, including decayed, missing and filled teeth (dmft); and decayed, missing and filled surfaces (dmfs) scores have been reported previously (Palmer et al., 2012b), and are summarized in Table 1. Dental rehabilitation therapy included application of 0.12% chlorhexidine gluconate to the gingiva and dentition using a sterile gauze to prepare the surgical area prior to beginning the procedure, followed by amalgam (Valiant® PH.D®), composite (Pulpdent® Etch-Rite 38% Phosphoric Acid Etching Gel, Optibond™, Z100™ and Filtek™ Supreme), and stainless steel crown restorations (3M ESPE, Unitek), formocresol pulpotomies (Patterson Dental), extractions, sealants (Patterson Dental), dental prophylaxis (NUPRO® prophylaxis paste [1.23% fluoride]), and sodium fluoride varnish (Cavity Shield™) application with a brush.

2.2 Plaque Sampling Procedure

For 1-year follow-up study described here, plaque samples were taken from each participant and compared to specimens collected at the three previous time points: 1) prior to the initiation of dental rehabilitation therapy, 2) within the 2-4 weeks post-rehabilitation visit, and 3) at the 6-month recall visit following dental rehabilitation.

2.3 Selection of Mutans Streptococci (MS) Isolates and Control Streptococci Strains

As described in Fazilat et al. (2010) and Palmer et al. (2012a), plaque specimens were plated on mitis salivarius agar (MSA; product number 229810, Difco, Becton, Dickinson and Company, Sparks, MD), supplemented with 1% sodium tellurite and the antibiotic bacitracin (0.2 Units/ml), to isolate MS. Colonies were grown on MSA plates for 48-72 hours (37°C, 5% CO2), and then individual colonies were selected based on typical MS morphology. Control streptococci strains include S. mutans ATCC strains 25175 and 35668, S. sobrinus ATCC 33478, and non-MS oral streptococci strain S. salivarius ATCC 13419.
Table 1: Demographics of Patients (G, J, K, L, and M) Returning for One-Year Recall Exam. This table was shown in part in Palmer et al. (2012a) and Palmer et al. (2012b) and is reproduced here with the kind permission of the American Academy of Pediatric Dentistry and CoAction Publishing, the publisher of the Journal of Oral Microbiology, respectively.

<table>
<thead>
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<th>Treatment Day</th>
<th>Sex</th>
<th>G</th>
<th>J</th>
<th>K</th>
<th>L</th>
<th>M</th>
</tr>
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<tbody>
<tr>
<td>Sex</td>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Treatment Age&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td>5 years</td>
<td>5 years</td>
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<td>5 years</td>
</tr>
<tr>
<td>Teeth Present&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>A-T</td>
<td>A-T</td>
<td>A-T</td>
<td>A-T</td>
<td>A-T</td>
</tr>
<tr>
<td>DMFT Score&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>11</td>
<td>13</td>
<td>18</td>
<td>13</td>
<td>12</td>
</tr>
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<td>DMFS Score&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>38</td>
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<td>41</td>
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<tr>
<td>Condition of Restorations</td>
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<td>Satisfactory</td>
<td>Satisfactory</td>
<td>Missing 1 restoration</td>
<td>Satisfactory</td>
<td></td>
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<tr>
<td>Number of New Carious Lesions</td>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lesion at Margin of Existing Restoration</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Treatment Age: Age of patient on day of full-mouth dental rehabilitation.

<sup>2</sup> Teeth Present: Teeth present on day of either full-mouth dental rehabilitation or at the 1-year recall exam. Letters denote primary teeth and numbers denote permanent teeth present following the Primary Universal Numbering System.

<sup>3</sup> DMFT: The sum of the primary teeth that is decayed (d), missing (m) or filled (f) due to dental caries.

<sup>4</sup> DMFS: The sum of the primary tooth surfaces that is decayed (d), missing (m) or filled (f) due to dental caries.

2.4 Genomic DNA Isolation, Conventional Polymerase Chain Reaction [PCR] and Arbitrarily Primed PCR (AP-PCR)

Genomic DNA was extracted from overnight liquid cultures, and *S. mutans* were independently identified using conventional PCR (Palmer et al., 2012a). Highly-specific primers for *S. mutans* and *S. sobrinus*, in addition to thermal cycling parameters, have been defined (Igarashi et al., 2000; Chen et al., 2007), and are also described in our previous reports (Palmer et al., 2012a; Palmer et al., 2012b). The amplification parameters for AP-PCR were similar to conventional PCR, with the exception of annealing at reduced temperatures (35°C for 30 seconds).

2.5 Xylitol-Susceptibility Assays and Statistical Curve-Fitting Analyses

MS genetic strains were grown overnight in BHI broth to ensure bacteria were in exponential growth-phase, prior to xylitol-susceptibility experiments. Cultures were then grown with various concentrations of xylitol, and growth curves were determined by measuring absorbance (A=600 nm) every hour from 0-10 hours, and finally at 24 hours. Independent cultures were treated with varying xylitol concentrations (0-5%). Xylitol concentrations were selected based on concentrations used in similar *in vitro* xylitol inhibition assays (Söderling et al., 2008) and are thought to bracket the effective dose of xylitol in saliva released from oral xylitol-containing products. Curve fitting analysis using cubic and quadratic models
(Raudenbush & Bryk, 2002; Hox, 2002; Skrondal & Rabe-Hesketh, 2004) was conducted to determine the xylitol concentration (w/v) that results in 50% inhibition of growth, using the absorbance value of the 0% xylitol control at peak logarithmic phase (typically at 9-10 hours) for normalization at 100%.

3 Results

3.1 Description of Study Participants

Nine patients were originally enrolled in this pilot study; seven (Patients G, H, I, J, K, L and M) were available for their initial 2-4 week recall visits, and only five patients (Patients G, J, K, L and M) made all appointments, including their 6-month and 1-year post-dental rehabilitation visits. Table 1 shows information collected from the five patients who completed all four sample collection visits. The pediatric dentistry patients were between the ages of 3 and 5 years old on the day of dental rehabilitation and in good health (American Society of Anesthesiologists [ASA] physical status I). The patients were all diagnosed with severe-early childhood caries (S-ECC) with dmft and dmfs scores ranging from 11-18 and 25-61 respectively (Palmer et al., 2012a) (see also Table 1). All patients in our study underwent full-mouth dental rehabilitation therapy under general anesthesia. They all also had antimicrobial chlorhexidine rinse, and fluoride varnish applied.

3.2 Identification of Mutans Streptococci Strains

Based on growth and colony morphology on mitis salivarius agar (MSA) and Gram stain analysis, up to 60 isolates were obtained from each plaque specimen originating from every patient at all collection periods, with each isolate confirmed as bacitracin-resistant, Gram-positive, oral streptococci. Using primers specific for S. mutans or S. sobrinus (both members of mutans streptococci, MS), and testing genomic DNA from isolates obtained from the seven patients (Patients G, H, I, J, K, L and M), we identified 37 genotypic strains of S. mutans, two strains [K2 and K3 strains] of S. sobrinus, and seven non-MS strains during the entire study (pre-rehabilitation, 2-4 week post-rehabilitation, 6-month post-rehabilitation and 1-year post-rehabilitation) (Palmer et al., 2012b), including the appearance of six new MS strains found only at the 1-year collection (Figure 1 and Table 2). For the five pediatric patients who completed the entire 1-year study, we identified 30 genotypic strains of S. mutans, two genotypic strains [K2 and K3 strains] of S. sobrinus, and five non-MS strains (Table 2; also Palmer et al., 2012b). Several of the new strains identified at 1-year post-dental rehabilitation were found only as single isolates, or were highly-related to other genotypes that differ by only single bands in their AP-PCR genetic profiles (Figure 1 and Table 2; e.g. compare L1c found only at the 1-year collection to dominant strain L1 and other minor MS strains L1a and L1b). Genomic DNA from several isolates were not amplified, or only weakly amplified using S. mutans-specific primers, and comprised seven additional genetic strains of bacitracin-resistant Gram-positive oral streptococci, which we termed non-MS strains. As described previously (Palmer et al., 2012a), these seven genotypic strains were subjected to 16S ribosomal RNA gene sequencing, which identified them as three strains of S. gordonii, three strains of S. anginosus, and one strain of Granulicatella adiacens, previously known as S. adjacens (Woo et al., 2003).
Figure 1: Genotypic strain diversity in pediatric dentistry patients at pre- and post-dental rehabilitation therapy (2-4 weeks, 6 months and 12 months). Each line represents distinct genotypes identified by AP-PCR. The dominant genotypes are marked in bold at each collection point (greater than 40% of the isolates). Dotted lines indicate the periods when genotypes were detected at one time point but were not detected at subsequent time points. Isolates were genotyped numerically within each patient (G, J, K, L and M). Only data for Patients G, J, K, L and M, who completed all recall visits, including the 12-month post-therapy visit, are included in this analysis. Portions of this figure were previously displayed in Palmer et al. (2012a) and in full in Palmer et al. (2012b) and were reproduced with the kind permission of the American Academy of Pediatric Dentistry and CoAction Publishing (the publisher of the Journal of Oral Microbiology), respectively.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Visit</th>
<th>Percentage of Genotypes</th>
<th>No. of Genotypes</th>
<th>Dominant Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>S. mutans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>G1  G2  G4  G4a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient G</td>
<td></td>
<td>Pre-Treatment</td>
<td>18  56  16  3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>0   98  0  0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-Treatment (6 months)</td>
<td>0   100  0  0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-Treatment (1 year)</td>
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<td>1</td>
</tr>
<tr>
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<td>13  56  7  2  0  16  4  0  2  0</td>
<td>7</td>
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<tr>
<td></td>
<td></td>
<td>Post-Treatment (2 weeks)</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-Treatment (6 months)</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-Treatment (1 year)</td>
<td>0   60  0  0  4  3  2  0  4  0  0</td>
<td>4</td>
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<td>Pre-Treatment</td>
<td>90  7  0</td>
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<td>Post-Treatment (1 year)</td>
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<td>2</td>
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<tr>
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<td></td>
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<td>Post-Treatment (6 months)</td>
<td>100  0  0  0  0  0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-Treatment (1 year)</td>
<td>89  0  6  3  2  0</td>
<td>4</td>
</tr>
<tr>
<td>Patient M</td>
<td></td>
<td>Pre-Treatment</td>
<td>14  44  0  2  0  0  0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-Treatment (4 weeks)</td>
<td>2   88  4  0  0  0  0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-Treatment (6 months)</td>
<td>0   40  0  0  12  0  0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-Treatment (1 year)</td>
<td>0   93  0  0  0  2  2</td>
<td>4</td>
</tr>
</tbody>
</table>

1 Genotypes confirmed as *S. mutans* by conventional PCR with *S. mutans* specific primers. Note that genotypes containing an “a”, “b”, or “c” suffix as in G4a or J2a and J2b differ from its matched comparison strains (in this case: G4 and J2) with the addition of one or more AP-PCR fragments (“a” suffix implies one additional band, “b” suffix implies two additional bands and “c” suffix implies three additional bands, all when compared to the AP-PCR profile of its matched genotypic strain). Note that individual MS genotypes were determined for comparison within each patient alone, and thus, comparisons of MS genotypes were conducted at only the intra-patient level. Please note that non-MS strains were also identified and published in Palmer et al. (2012a), but not reflected in this table for space considerations, and that the genotype percentages were calculated based on the sum total of all MS and non-MS genotypes observed at each time collection point.

2 Genotype K1 isolates obtained at the 2 week post-treatment collection did not yield robust PCR products using *S. mutans*-specific primers and conventional PCR; however, these isolates were defined in the MS group because they retained identical AP-PCR fingerprints when compared to other K1 isolates.

Note: This table was shown in part in Palmer et al. (2012a) and Palmer et al. (2012b) and is reproduced here with the kind permission of the AAPD and CoAction Publishing, the publisher of the *Journal of Oral Microbiology*, respectively.

**Table 2:** Identification and Percentage of MS and Oral Streptococci Genotypes at Each Visit.
3.3 Genotypic Strains Remain Dominant and New Minor Strains Appear at 1-Year Post-Rehabilitation

The numbers of genotypic strains identified from any one patient over the entire collection period ranged from 3-9, or from any one visit from 3-7 (Figure 1 and Table 2). In all five patients who completed all recall visits, including the 1-year recall, the highest number of strains was observed at the pre-treatment visit, and dominant strains representing >44% of the population examined emerged at post-rehabilitation collections. Four out of these five patients (Patients G, K, L, and M), had the same dominant genotypic strain at 6 months post-rehabilitation as at 1-year post-rehabilitation (Figure 1 and Table 2). Interestingly, in four patients (Patients J, K, L and M), the number of MS strains became more diverse at 1-year post-rehabilitation, with the emergence of new minor strains. Also, in almost all cases and collection times (with the exception of Patient J), there was only one dominant strain, each representing >56% of the strain population examined (Figure 1 and Table 2). In Patient J, genotype J2 was the dominant MS strain at pre-dental rehabilitation, disappearing (perhaps below detection) during the 2-week and 6-month collections, and then reappearing as the dominant strain at 1-year post-rehabilitation. Genotype J3 remained throughout the entire sampling period, and was the dominant MS strain during the 2-week and 6-month post-rehabilitation sampling times. The genotypic distribution patterns for the patient cohort were distinctive and unique for each patient; all comparisons of genotypes were conducted with strains collected within each individual patient, and not between patients. Inter-patient comparisons of MS strains were not conducted as part of this study.

3.4 Xylitol-Susceptibility of Dominant and Minor MS Genetic Strains

Using an in vitro growth assay, we determined the 50% xylitol inhibition values for select MS strains ranged between 2.48% to 33.3% xylitol, using the absorbance value of the control 0% xylitol group at peak logarithmic phase (typically at 9-10 hours) as the 100% normalization value for each strain (Figures 2 and 3). S. mutans ATCC strains 25175 and 35668, generally considered to be laboratory attenuated strains, had very similar 50% xylitol inhibition values of 3.35% and 3.30%, respectively (Figures 2 and 3). The majority of the MS genetic strains analyzed (15 out of 23 strains from Patients G, J, K, L and M) exhibited 50% xylitol inhibition values ranging from 2.48% - 5.58%, similar to the two S. mutans ATCC control strains. In patients where 1-year post-dental rehabilitation specimens were collected, dominant strains G2, J3, K1, L1, and M3 exhibited 50% xylitol inhibition values of 2.95%, 3.26%, 3.45%, 7.06%, and 3.86%, respectively (Figures 2 and 3). In Patient J, dominant strain J3 at 6 months post-rehabilitation therapy was highly xylitol-resistant with a 50% xylitol inhibition value of 33.3%, but was replaced as the dominant strain at 1-year post-therapy by dominant strain J2, which displayed typical xylitol-susceptibility (Figure 2). In the five pediatric patients who completed all post-rehabilitation recall visits [Patients G, J, K, L and M], the dominant MS genotypic strain at the 1-year post-rehabilitation collection exhibited 50% xylitol inhibition values similar or close to the values retained by the S. mutans ATCC control strains.

4 Discussion

Dental caries is one of the most common chronic diseases found throughout the world. Dental caries results from multifactorial and complex interactions at the tooth surface between certain oral bacteria and
Figure 2: Xylitol susceptibility curves for dominant MS strains and *S. mutans* ATCC 25175. Dominant MS strains G2, J2, J3, K1 and L1, and *S. mutans* ATCC 25175 were propagated in BHI for 24 hours at 37°C, and subsequently diluted in fresh BHI to an absorbance (600 nm) level of 0.1 to initiate logarithmic growth in the presence or absence of xylitol (final concentrations of 0%, 0.001%, 0.01%, 0.1%, 1% and 5% xylitol). Cultures were measured spectrophotometrically every hour for 10 hours and then at 24 hours, using four replicates per time point for each xylitol concentration. Plots were constructed and then curve fitted using cubic or quadratic models (Raudenbush & Bryk, 2002; Hox, 2002; Skrondal & Rabe-Hesketh, 2004) to determine the theoretical xylitol concentrations for 50% inhibition of growth, using the peak absorbance of the 0% xylitol control as the normalization factor at 100%. This figure was previously displayed in Palmer et al. (2012b) and was reproduced with the kind permission of CoAction Publishing (the publisher of the *Journal of Oral Microbiology*).
Figure 3: Bar graphs illustrating xylitol concentrations (w/v) for 50% inhibition of growth for dominant MS strains and select minor MS strains from Patients G, J, K, L and M. 50% inhibition values are also displayed for \textit{S. mutans} ATCC 25175 and 35668. Dominant strains are in bold. Xylitol concentrations (w/v) for 50% inhibition of growth for: 1) \textit{S. mutans} ATCC 25175 and 35668 are 3.35% and 3.30%, respectively; 2) Strains G1, G2, G3, G4, and G4a are 5.58%, 2.95%, 17.4%, 2.48% and 14.1%, respectively; 3) Strains K1 and K2 are 3.45% and 3.61%, respectively; 4) Strains J1, J2, J2a, J2b, J2c, J3, J3a, and J4 are 3.35%, 3.26%, 3.77%, 4.28%, 9.05%, 33.3%, 3.65% and 4.12%, respectively; 5) Strains L1, L1a, L2 and L3 are 7.06%, 7.00%, 3.35% and 3.43%, respectively; and 6) Strains M1, M3, M3a and M4 are 7.07%, 3.86%, 4.10% and 21.7%, respectively. M3 was the dominant strain in Patient M throughout the entire 1-year collection period. M1 was an additional co-dominant strain in Patient M at 6 months post-rehabilitation therapy and was defined as a non-MS strain (Palmer \textit{et al.}, 2012a). This figure was previously displayed in Palmer \textit{et al.} (2012b) and was reproduced with the kind permission of CoAction Publishing (the publisher of the \textit{Journal of Oral Microbiology}).

their products, salivary constituents, and dietary carbohydrates. Many bacteria have been implicated in caries formation, but research has shown that \textit{S. mutans} is essential in the pathogenesis of dental caries. \textit{S. mutans} is thought to be highly cariogenic because of multiple virulence factors including acidogenicity and acid tolerance, and its ability to generate copious biofilms made of insoluble extracellular glycans. Distinct strains of \textit{S. mutans} produce differential levels of these virulence factors, including differing amounts of glucosyltransferase enzymes (Alaluusua \textit{et al.}, 1996). Further, caries-active individuals have been shown to often harbor larger numbers of MS genotypes with increased capacity to synthesize water-insoluble glycans, which are essential for extracellular matrix production in dental plaque biofilms (Namimoga \textit{et al.}, 2005). Thus, this genetic diversity among the various \textit{S. mutans} genetic strains, with their corresponding differences in virulence gene expression must now be taken into consideration when thinking about the overall pathogenesis and epidemiology of dental caries.

We originally designed our pilot study to begin to understand this genetic diversity of \textit{S. mutans} and how it is displayed in a small group of children with S-ECC. We also wanted to determine if any changes occurred in the genotypic population within each of these patients following full-mouth dental rehabilitation therapy. Prior to treatment these children with S-ECC harbored multiple different strains of
MS (i.e., *S. mutans* and *S. sobrinus*), whose relative numbers changed dramatically after treatment (Palmer *et al.*, 2012a). Continuing these studies, using the remaining members of the original patient cohort, we have demonstrated in our primary article (Palmer *et al.*, 2012b) and also now show the continued presence of dominant MS genotypic strains and the emergence of additional minor MS strains at 1-year post-dental rehabilitation. We also describe the *in vitro* xylitol resistance of dominant and select minor MS genotypic strains.

### 4.1 Genetic Analysis and Identification of MS Strains

Investigations by several research groups, including Napimoga *et al.* (2005), Lembo *et al.* (2007), and Baca *et al.* (2008) have validated the use of arbitrarily primed-PCR (AP-PCR) in discriminating MS genotypes within individuals, and have formed the basis of both our original pilot study, and the studies outlined here. We also now find that AP-PCR profiles of MS isolates obtained at 1-year post-rehabilitation therapy can be reproducibly and reliably compared to profiles of MS isolates obtained earlier in the original study. Thus, AP-PCR and the genetic profiling of MS strains allow longitudinal epidemiology studies to be conducted over the 1-year post-rehabilitation therapy period.

### 4.2 MS Colonization and Genotypic Diversity in Children with Severe Early Childhood Caries

Mitchell *et al.* (2009) have suggested that the composition of strains within the MS population, or the acquisition and loss of specific MS strains, is a dynamic, active process in S-ECC patients. We also observed similar MS population shifts in patients described in our study, for all time periods including 1-year post-rehabilitation. As described in our previous reports (Palmer *et al.*, 2012a; Palmer *et al.*, 2012b), the re-appearance of genotype J2 at 1-year post-rehabilitation in Patient J, as well as the appearance of new minor MS strains in four patients (Patients J, K, L and M) at 1-year post-rehabilitation, may be due to re-infection from external sources. Alternatively, genotype J2, undetected at the 2-4 week and the 6-month collections, may have been present at numbers below the threshold of our detection. In the case of Patient J, and other individuals with S-ECC, it is probable that MS genotypes were acquired either vertically from their mother or horizontally from other members of their family or extended care group. The diagnoses of ECC and S-ECC are associated with several risk factors including caregivers with high levels of MS and untreated carious lesions, frequent ingestion of sucrose-rich diets, and poor oral hygiene practices. In combination, these factors can result in MS colonization at earlier ages, with higher bacterial levels and greater number of MS genotypes than in caries-free children (Napimoga *et al.*, 2005; Napimoga *et al.*, 2004; Lembo *et al.*, 2007; Baca *et al.*, 2008; Mitchell *et al.*, 2009; Poggio *et al.*, 2009). They may also predispose these patients to easier re-colonization by MS strains after caries treatment.

As described here and in our previous report (Palmer *et al.*, 2012a), our enrolled children with S-ECC, ages 3-5 years, exhibited 3-7 MS genotypes prior to dental rehabilitation therapy, and most exhibited only 1-3 genotypes 6 months post-therapy. Single dominant genotypes, were identified in four of five pediatric patients 6-months post-rehabilitation therapy. By 1-year post-therapy however, the diversity of MS genotypes increased in four out of five patients [e.g.: Patients J, K, L and M], with the appearance of six new minor MS strains.

Our primary objective in these studies was to define changes that occur in the composition of MS genetic strains in caries-active children, after full-mouth dental rehabilitation therapy. Collectively our results are most similar to those of Peralisi *et al.* (2010), where we observe a wide diversity of genetic MS strains in children with S-ECC prior to treatment, but we also demonstrate that dental rehabilitation therapy results in the appearance of single dominant MS strains by 6-months post-therapy. Unfortunately
we were unable to determine if the reduction of MS strains and appearance of single dominant strains were due to the selective effects of the antimicrobial rinse, application of fluoride varnish, the restorative procedure itself, or a combination of these therapies. We also found that by 1-year post treatment, genetic diversity of MS strains again increased within most of the S-ECC patients studied, perhaps indicative of a return to circumstances more similar to pre-treatment within these individuals’ mouths.

4.3 Full-Mouth Dental Rehabilitation Therapy and Effects on Diversity of MS Strains

In our original study (Palmer et al., 2012a; Palmer et al., 2012b), we examined the diversity of genotypic strains of MS and other non-MS streptococci from seven pediatric patients who had S-ECC, with an additional objective of evaluating the effects on these MS strains of the standard regimen for full-mouth dental rehabilitation therapy. In most patients, dental rehabilitation therapy reduced the diversity of oral MS from many genotypic strains to only 1-2 dominant strains by 6 months post-therapy. We presume that the treatment of the carious lesions, as well as the antimicrobial rinse and fluoride treatment reduced the total bacterial numbers of all strains immediately following dental rehabilitation, but the effectiveness of the therapy was time-limited, with dominant strains appearing by 6 months post-rehabilitation. In most of the patients examined, and as described in our previous reports (Palmer et al., 2012a; Palmer et al., 2012b), dental rehabilitation therapy eliminated several non-MS strains and allowed specific strains of highly acidogenic *S. mutans* bacteria to become dominant strains. Finally as shown here, new minor MS strains appeared at 1-year post-therapy, as a result of re-infection from the primary care giver or other external sources.

Our studies do not address how patient compliance with at home dental care instructions might have impacted the appearance of the MS dominant strains by 6-months, or the appearance of new minor strains by 1-year post-rehabilitation therapy. This of course is an area of research that needs to be examined further.

We understand that we have limited numbers of patients in both the pilot study (Palmer et al., 2012a) and in the 1-year follow-up study (Palmer et al., 2012b), but believe that this work is statistically substantiated by Cheon et al. (2011), who have used probabilities to determine the minimum number of MS isolates from an individual required to fully evaluate diversity of bacterial genotypes. Cheon et al. (2011) determined theoretically that screening seven MS isolates from any one specimen collection was sufficient for the detection of up to four MS genotypes with a success probability of 78%. In our studies we screen at least ten MS isolates per oral specimen, thus increasing the probability of identifying all MS strains present.

4.4 Xylitol-Susceptibility of MS Strains

One commonly used caries prevention modality is xylitol. Even though xylitol has been studied for decades, its use as a caries preventive tool is still controversial. Even so many pediatric dentistry organizations throughout the world, including The American Academy of Pediatric Dentistry, have officially recognized the benefits of xylitol as a caries preventative measure for use in children. Therefore, we sought to determine the *in vitro* xylitol susceptibility of the some of the MS strains, including the dominant strains, isolated from our S-ECC patients.

Our xylitol inhibition experiments indicate that most of the dominant MS strains are similar in xylitol resistance to the attenuated *S. mutans* ATCC control strains, with some strains being variably inhibited by xylitol *in vitro*. Moraes et al. (2011) indicate that xylitol (40% solution) may not significantly suppress *S. mutans* counts or plaque accumulation in young children, as opposed to adults, potentially
implicating the existence of xylitol-resistant MS strains. Our studies support Moreas et al. (2011) and affirm the existence of MS strains showing variable inhibition, including strains dramatically more xylitol-resistant. The presence of these xylitol-resistant MS strains would no doubt impact the effectiveness of any xylitol preventive treatments in individuals.

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