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Diversity, Commonality, and Stability of Mutans Streptococci in High Caries Risk Children

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DIVERSITY, COMMONALITY, AND STABILITY OF MUTANS STREPTOCOCCI IN HIGH CARIES RISK CHILDREN

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ABSTRACT

Mutans streptococci (MS), *Streptococcus mutans and Streptococcus sobrinus* are associated with the initiation of dental caries in humans. Initially, a single *S. mutans* genotype has been shown to colonize primary molars soon after eruption. More genotypes can be acquired with age and further dental development. Current methods used to genotype isolates from an individual have not systematically determined the minimum number required to demonstrate an individual's genetic diversity. The purpose of this study was to determine the optimum number of oral isolates of MS (i.e., from plaque samples) to genotype in order to demonstrate diversity. This study also initiated a longitudinal study of *S. mutans* genotypes to determine diversity, commonality, and stability for primary and newly erupting permanent molars in a high caries risk population. The subjects included 5-6 year old children (index children) prior to eruption of at least one permanent molar and adults. Cultured MS positive isolates were verified by SYBR PCR. DNA was extracted for repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) followed by Microfluidics-based DNA amplicon fractionation using an Agilent 2100 Bioanalyzer. Data analysis was performed with the DiversiLab software (v3.3) using the Pearson Correlation Coefficient method to create DNA fingerprints, dendrograms, and similarity matrices to interpret data. This study showed that 7 to 10 isolates were determined to be a practical number of isolates to collect per individual (i.e., up to 51 to 78% probability of identifying 4 genotypes when they exist). Nineteen children were found to

have a total of 11 genotypes at baseline and at 6 month follow-up with a mean of 1.5 genotypes (diversity) per individual. The most common genotype was shared by six children, and seventeen among nineteen children shared at least one genotype with another child's plaque (commonality). A predominant genotype from each individual (N = 19) at baseline was retained at the 6 month follow-up (stability = 100%). This study demonstrated the characteristics of diversity, commonality, and stability of *S. mutans* genotypes among a high caries risk children population in a 6 month longitudinal study. The method of highly automated rep-PCR with DNA chip assay is an efficient tool to characterize *S. mutans* genotypes and constructing a longitudinal database.

Keywords: *S. mutans,* children, plaque, rep-PCR, diversity, commonality, stability

DEDICATION

This thesis is dedicated to my husband, Dr. Ho-wook Jun who constantly encouraged me to step up in every moment; my parents and parents-in-laws in South Korea who have prayed for me all their life; and my lovely children Joseph and Claire who have given me a happy smile every day.

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GENETIC DIVERSITY OF PLAQUE MUTANS STREPTOCOCCI WITH REP-PCR

CHARACTERIZATION OF *Streptococcus mutans* GENOTYPES IN HIGH CARIES RISK CHILDREN

LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AP-PCR	Arbitrarily-Primed Polymerase Chain Reaction
DMF	decayed, missing, filled
dmf	for deciduous teeth decayed, missing, filled
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
G	genotype
HCl	hydrochloric acid
IRB	Institutional Review Board
LB broth	Luria-Bertani broth
MLEE	multi locus enzyme electrophoresis
MLST	multilocus sequence typing
MS	Mutans Streptococci
MS agar	mitis salivarius agar
MSB agar	mitis salivarius agar with sucrose and bacitracin
N	number
NHANES	National Health and Nutrition Examination Survey
NIH	National Institute of Health
PFGE	Pulsed-field gel electrophoresis

PMs	permanent molars
RAPD	Randomly Amplified Polymorphic DNA
rcf	relative centrifugal force
rep-PCR	repetitive extragenic palindromic polymerized chain reaction
SD	standard deviation
TE buffer	Tris and EDTA buffer
TH	Todd Hewitt
THB	Todd Hewitt Broth
UPGMA	unweighted-pair group methodology with arithmetic mean

GENERAL INTRODUCTION

Dental caries in American children has continued to decline significantly over the past two decades. However, caries is prevalent in certain populations of children where access to dental care is poor. According to the National Health and Nutrition Examination Survey III data, untreated dental caries prevalence is as high as 45.2% in 6- to 8-year old children in the socioeconomic group below the federal poverty level (NIDCR/CDC, 2002). A natural history of the development of caries, including the relative contributions of genetic and environmental factors to caries of the primary and permanent dentitions, has yet to be clearly characterized. Understanding the mechanisms of developing dental caries requires studying the complex interaction of oral microbes, host, and substrate (Liljemark and Bloomquist, 1996; Morhart and Fitzgerald, 1976; Sbordone and Bortolaia, 2003). As an oral microbial factor, the mutans streptococci (MS), *Streptococcus mutans* and *Streptococcus sobrinus*, have been well studied since first detected by J. Kilian Clarke (Clarke, 1924). MS are reviewed as the primary etiological agents associated with the initiation of dental caries in humans (Hamada and Slade, 1980; Koga *et al*., 1986; Loesche, 1986). These Gram positive bacteria adhere to tooth surfaces for colonization, and flourish in an acidic environment.

Caries is an infectious and transmissible disease (Alaluusua et al., 1996; Berkowitz, 2003; Li and Caufield, 1995; Loesche, 1986; Seow, 1998; Tanzer et al., 2001). By means of genetic techniques to identify the MS genotype, it has been reported that many young children appear to be infected with the MS by vertical transmission from their

mothers (Berkowitz and Jordan, 1975; de Soet *et al*., 1998; Emanuelsson *et al*., 1998; Gronroos *et al*., 1998; Klein *et al*., 2004; Kozai *et al.*, 1999; Li and Caufield, 1995; Lindquist and Emilson, 2004). However, other studies have demonstrated that MS genotypes can also be acquired during dental development by horizontal transmission from peers (Kozai et al., 1999; Liu et al., 2007; Mattos-Graner et al., 2001; Mitchell et al., 2009).

The observed genotypic features from previous studies can be characterized as studies to investigate "diversity", "commonality", and "stability" of MS individually or collectively. In this thesis, diversity is defined as the number of genotypes found within an individual, family, or population. Commonality is defined as a measure of the degree that genotypes are shared among a group, family or population. Depending on the diversity of MS, the commonality may provide a scientific clue explaining transmission of MS within a group. Stability is defined as the persistence of genotypes over time, and may provide important information related to the predominance of genotypes longitudinally. These three genotypic characteristics potentially will provide data to support for inferences related to transmission of *S. mutans* and the etiology of dental caries.

To evaluate these unique features of MS, several phenotyping and genotyping techniques have been introduced and investigated. A variety of methods have been developed for this approach, each with advantages and disadvantages. The more involved multilocus sequence typing (MLST) (Soll *et al*., 2003) is labor intensive and costly. Ribotyping (Alaluusua *et al*., 1994) and PCR ribotyping (Lagatolla *et al*., 1996) have been used, but often result in difficulty distinguishing among different subspecies. Randomly amplified polymorphic DNA (RAPD) (Redmo Emanuelsson *et al*., 2003; Truong *et al*., 2000) has low reproducibility due to short random primers and a low annealing tempera-

ture, while amplified fragment length polymorphism (AFLP) generates DNA profiles that are difficult to interpret (Janssen *et al.,* 1996). Pulsed-field gel electrophoresis (PFGE) has been shown to be a valuable epidemiologic tool with good intra and inter-laboratory reproducibility, but is labor intensive (Jordan and LeBlanc, 2002; Tenover *et al*., 1995). Arbitrarily primed polymerase chain reaction (AP-PCR) has demonstrated comparable results to the chromosomal DNA fingerprinting, but is not reproducible due to its low stringency condition (Li and Caufield, 1998; Welsh and McClelland, 1990). Repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) has been introduced as an effective method for bacterial strain typing with high reproducibility and high throughput, which are essential for longitudinal study (Alam *et al*., 1999; Louws *et al*., 1999; Versalovic *et al*., 1991).

Our laboratory began a longitudinal study of *S. mutans* genotypes using PFGE; but in order to manage the large number of isolates for analysis (> thousands), we investigated the use of rep-PCR. When compared to other methods, rep-PCR results may be obtained in less than 10 hours after colonies are grown, while PFGE results take 5 days (Bou *et al*., 2000). Furthermore, with rep- PCR, as little as a 1 µL of DNA sample can be fractionated and bound with an intercalating dye, then passed over a laser creating a graph of fluorescence intensity over time in the channel of a Microfluidics LabChip® (Healy *et al*., 2005). Briefly, rep-PCR is an improved genotyping tool in terms of broad utilization and reproducibility while reducing experimental time and costs. The highly integrated rep-PCR includes thermal cycling parameters that incorporate microfluidics based DNA amplicon fractionation and detection. The resulting graph translates into fingerprint profiles showing multiple bands of varying sizes and intensities that can be in-

terpreted on an internet based analysis system (Healy *et al.*, 2005; Moser *et al*., 2010; Svec *et al*., 2008). Therefore, we have used the rep-PCR method to explore genetic diversity among children and their family members with an automated DNA Chip assay. Reported and stored data have demonstrated remarkable reproducibility of microbial DNA genotyping, regardless of various sample collection time frames.

One challenge in this line of investigation is determining how many isolates are required from oral samples to be representative of the genetic diversity within an individual. We have constructed probability estimates (see manuscript, Fig. 1) to identify the minimum number of *S. mutans* isolates for required reliably evaluating of diversity, stability, and commonality of *S. mutans* genotypes in high caries risk populations. Furthermore, this study has initiated the construction of a *S. mutans* genotype library. Therefore, the database will function as an extensive source to understand the infectious nature of dental caries, and serve as the foundation for developing a caries prevention strategy for high caries risk children. The thesis includes two different purposes for the studies. One is published in the Journal of Dental Research as the "Genetic Diversity of Plaque Mutans Streptococci with rep-PCR". The other study is the "Characterization of *Streptococcus mutans* Genotypes in High Caries Risk Children" which will be finalized for submission to a peer-reviewed journal at a later date.

GENETIC DIVERSITY OF PLAQUE MUTANS STREPTOCOCCI WITH REP-PCR

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ABSTRACT

Mutans streptococci (MS) are key organisms associated with the etiology of dental caries. Using probabilities that were tested by oversampling, we designed this study to determine the minimal number of MS isolates from an individual required to evaluate diversity of genotypes. MS isolates were genotyped by repetitive extragenic palindromic-polymerase chainreaction (rep-PCR). Analysis of 20 isolates from individuals resulted in a mean of 1.6 and 2.4 genotypes in children (N = 12) and adults (N = 10), respectively. In a follow-up study, reducing the number of isolates to 7-10 resulted in a theoretical probability of up to 78% for detecting up to 4 genotypes. A mean of 1.5 genotypes was found in 35 children and 10 adults. These findings provide evidence for the design of studies of MS genotyping that can serve as a model for the analysis of genotypes within individuals.

Key words: *Streptococcus mutans*, microbial genetics, caries, biostatistics, Plaque/Plaque biofilms

INTRODUCTION

The incidence of dental caries in the U.S. has declined significantly since 1970. Widespread fluoridation has been credited for the majority of this reduction, nevertheless 70% of children develop active caries by age 17 (Marthaler, 2004). Caries is especially prevalent in certain children from population demographics where access to dental care is poor.

As a means of understanding the infectious nature of caries, the concept of diversity was introduced. "Diversity" refers to the number of genotypes (or phenotypes, *i.e*., bacteriocin-types) within an individual. To identify diversity of the MS group, investigators have used numerous typing methods. Examples of MS and other eubacteria genotyping techniques previously utilized include genomic analysis, pulsed-field gel electrophoresis (PFGE), and polymerase chain reaction (PCR) based genomic fingerprinting. PFGE provides useful genotypic profiles, and is considered the gold standard for strain level identification but is labor intensive (Alam *et al*., 1999). One PCR method, repetitive extragenic palindromic-PCR (rep-PCR), amplifies many uniquely sized amplicons representing regions between target non-coding, repetitive sequences in the genome (Healy *et al*., 2005). An advantage is that rep-PCR requires as little as 50 ng of DNA to rapidly produce highly specific, sensitive, and consistent gene profiles. Thus, this method provides an efficient means to analyze diversity of MS among populations in longitudinal research (Moser *et al*., 2010).

A primary challenge in the assessing oral genotypes for an individual who may have multiple genotypes is to determine how many bacterial isolates are required from the individual to provide a representative sampling of the genetic diversity. Numerous genotyping experiments have indicated that most children have fewer than 3 MS genotypes and adults have less than 4. (Emanuelsson et al., 1998; Kozai et al., 1999; Mattos-Graner et al., 2001). However no studies have systematically documented the minimum numbers of isolates required from each individual for an accurate demonstration of the individual's genetic diversity.

The purpose of this study was to determine the number of genotypes present in an individual by over sampling (*i.e.*, 20 isolates per individual) from a group of children and adults. A genotyping method based on rep-PCR was selected because of its potential to determine not only the optimized numbers of isolates per individuals, but also to allow for the construction of an extensive MS gene library.

MATERIALS AND METHODS

Participants & Sample Collections

The sample population was a high-caries-risk community in Perry County, Alabama, with no regular access to dental care for children. The study population was drawn from children from kindergarten classes (5-6 years old) of a public elementary school. Samples were also collected from a group of adults in this community, who may or may not have been related to the children in this study. The study was approved by the Uni-

versity of Alabama at Birmingham (UAB) Institutional Review Board with participants and parents of children providing assent (children) and informed consent (parents).

Power Calculations

The study was designed to provide information on the number of specific genotypes from plaque of individuals. Using probability estimates (Figure 1) based on the assumption that all genotypes are equally distributed among a sample (*i.e.*, such as a plaque sample), then each genotype (k) is equally likely to be collected and thus to identify each of k genotypes from n samples the probability (P) is:

$$\text{Prob} = 1 - (-1)^{(j+1)} \left[\sum_{j=1}^{k-1} \frac{k!}{(k-j)!j!}\right] \left(\frac{k-j}{k}\right)^n$$

Thus, from this equation, it was determined that 20 isolates would be sufficient to provide 99% power of identifying up to 4 genotypes. With 20 isolates, the probability of observing at least 5 genotypes, if they exist, is 94% and thus, we had ample power to assess whether there were more than 4 genotypes likely to be present.

Isolation of mutans streptococci (MS)

We used mesial, distal, buccal, lingual, and occlusal surfaces of multiple primary and/ or permanent molars of children were used to collect pooled plaque samples using sterile tooth picks and subsequently transferred to 1 mL of reduced transport medium (Syed and Loesche, 1972). Adult plaque samples were pooled from permanent molar teeth. The plaque samples were stored on ice while transported 100 miles to the UAB School of Dentistry in Birmingham, AL. The plaque samples were processed within 24

hours of collection by vortexing and sonication (Vibra Cell, Sonics & Materials Inc., Newtown, CT, USA) for 30 seconds on ice at amplitude of 50. Samples were serially diluted in 0.05 M potassium phosphate buffer and plated on Mitis Salivarius agar (Difco /Beckton Dickinson, Sparks, Maryland, USA) containing 200 unit/liter of Bacitracin and 20% sucrose (Gold *et al*., 1973). Bacterial plating was performed with a Spiral Plater (Spiral System™ Inc, Cincinnati, OH, USA). Culture plates were incubated at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) for 48 hours (Lennette *et al*., 1974).

Individual MS colonies were selected based on morphological appearance for inoculation into Todd-Hewitt Broth (THB) (Beckton Dickinson, Sparks, Maryland, USA). Isolates were incubated for 24 hours under anaerobic conditions, and then stored at $^{-}80^{o}$C for future processing. Twenty frozen MS isolates *per* individual were plated on to Todd-Hewitt Agar (THA) and grown anaerobically at 37ºC for 48 hours. Isolated colonies were then inoculated into THB and incubated anaerobically for 18-24 hours. All isolates were confirmed MS prior to genotyping by means of SYBR Green real time PCR according to a method described previously (Yoshida *et al*., 2003).

Extraction of DNA for rep-PCR

The THB inocula were centrifuged at 2800 rcf for 20 minutes to pellet cells. THB was removed, and cells were washed by re-suspension in 1 ml of TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 7.6) followed by centrifugation at 21,000 rcf for 5 minutes. TE buffer was removed and final cell pellets were saved at -20°C until ready to be extracted. DNA was extracted with the Ultra Clean™ Microbial DNA Isolation Kit. (MoBio Labor-

atories, Carlsbad, CA, USA). Quantitation of DNA was performed by a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA). We controlled the quality of DNA by maintaining a minimum value of 1.7 and 0.9 respectively for 260/280 ratio and for 260/230 (an estimate of protein contamination).

rep-PCR and DNA Chip application

Rep-PCR was performed with DiversiLab streptococcus kit (bioMerieux, Durham, NC, USA) and ABI9700 (Applied Biosystems, Foster City, CA, USA) with a gold block according to the following parameters: initial denaturation of 94ºC for 2 min; 35 cycles of denaturation at 94ºC for 30 seconds; annealing at 50ºC for 30 seconds; extension at 70ºC for 90 seconds; and a final extension at 70ºC for 3 minutes. PCR reaction volume was a total 25 µL including 2µL of 25-45 ng/µL of purified genomic DNA. DNA amplicons from rep-PCR were separated on microfluidics chips and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA.) with the DNA Chip kit (bioMerieux, Inc., Durham, NC, USA) (Bou *et al*., 2000; Healy *et al*., 2005). In addition to sample DNA, a molecular-weight ladder and Ingbritt C (*S. mutans* positive control) were run on each chip to ensure consistency of results, similar to the method which used in AP-PCR analysis of MS (Li *et al*., 2001). A negative control was run on the first chip processed for each rep-PCR batch of samples to ensure absence of contamination in the PCR.

Analysis of DNA Fingerprints

Virtual gel images of each strain were generated by DiversiLab v 3.3 software (bioMerieux, Inc., Durham, NC, USA) and the Pearson correlation coefficient method was used to determine distance matrices for similarity calculation of DNA band intensity and location. The unweighted-pair group methodology with arithmetic mean (UPGMA) was utilized to create dendrograms, which provided a hierarchical cluster representation of similarities between samples and indicated strain level groupings of the MS. The criteria used to determine distinct genotypes were defined by rep-PCR results that differed based on major or minor banding differences (*i.e*., greater than or less than 100 fluorescence units, respectively). If two isolates differed by one major band or more than two minor bands, they were distinct (Moser *et al*., 2010). The DiversiLab website stored the reports that were generated and included dendrograms, virtual gel image, electropherograms, and scatter plots to aid in interpretation of the data.

Follow-up Study

Based on the initial analysis of 20 isolates from 12 index children and 10 adults, further analyses were done with samples from 35 additional children and 10 adults with a smaller number of isolates from each plaque sample, *i.e*., 7-10 isolates.

RESULTS

All isolates used for rep-PCR were confirmed to be *S. mutans* by SYBR Green PCR (Yoshida *et al*., 2003). Representative rep-PCR results illustrating the diversity of genotypes of the individuals are shown in Figure 2. Table shows the results of rep-PCR

analyses: Twelve children samples with 20 isolates from plaque samples resulted in the observation of 5 children who had only 1genotype (42%), while seven had 2 (58%) genotypes (mean = 1.6 genotypes). Three of 10 adults had a single genotype (30%), two had 2 genotypes (20%), four had 3 genotypes (40%), and one had 5 genotypes (10%) (mean = 2.4 genotypes).

The predicted numbers of isolates required for the identification of different numbers of genotypes, assuming the MS are equally distributed in the dental plaque in the oral cavity, are illustrated in Fig. 1. Based on these calculations, the results from the analyses carried out with 20 isolates *per* sample in children and adults-that is, analysis of 20 isolates- therefore, constituted oversampling for the group tested. Further, 7 to 10 isolates were determined to be a reasonable number of isolates to collect per individual (*i.e.*, up to 51 to 78% probability of identifying 4 genotypes when they exist). Thus, based on the findings from the first 12 children and 10 adults, additional analyses of isolates from plaque samples collected from 35 additional children and obtaining 7 to 10 MS isolates *per* child (385 total isolates) were analyzed by rep-PCR. This analysis resulted in identification of 22 children that had a single genotype (63%), 10 had 2 genotypes (29%), 1 had 3 genotypes (3%) and 2 children had 4 genotypes (6%) (Table). The average number of genotypes from this sample was 1.5 *per* child. Ten additional adults had plaque samples collected, yielding 7-10 MS isolates (88 total isolates) that were analyzed by rep-PCR. Six of these were found to have 1 genotype (60%), three had 2 genotypes (30%), and one had 3 genotypes (10%) with an average of 1.5 genotypes per subject. No differences were found between 20 and 7-10 isolates with Fisher's exact test in children ($p = 0.3111$) and in adults ($p = 0.3698$).

DISCUSSION

Rep-PCR has been introduced to genomic analysis with numerous benefits over PFGE and AP-PCR. PFGE, which uses restriction enzymes to digest the genome, gel electrophoresis and pattern analysis by direct DNA staining or DNA hybridization, requires long run times and thus is best only when there are limited sample numbers (Louws *et al*., 1999). AP-PCR has been utilized to demonstrate the diversity of bacterial genotypes in several scientific fields using arbitrary primers which amplify variable-size PCR products, but due to problems with reproducibility is not practical for a large longitudinal population study to verify genotype characteristics (Bou *et al*., 2000; Moser *et al*., 2010). However, rep-PCR effectively and consistently generates genotypic profiles using a nanogram scale of genomic DNA and is able to compare data longitudinally and potentially between laboratories (Healy *et al*., 2005; Louws *et al*., 1999). The findings presented herein demonstrated the usefulness of rep-PCR in identifying genotypic differences of MS (*S. mutans)* isolated from children and adults plaque.

The primary objective of this study was to determine the minimal number of isolates required per individual for rep-PCR analysis to demonstrate diversity of MS in a population at a time point and be confident that more types are not missed. The first approach to this study focused on using 20 isolates *per* individual to screen the pertinent number of genotypes. From probability computations, assuming five or less genotypes per individual, 20 isolates were considered to be oversampling of this group of individuals. By oversampling (*i.e*., 20 isolates), adults were found to have more diversity than children, with a range of 1-5 genotypes and mean of 2.4 genotypes *per* person. Therefore, 20-isolate analyses found that at most 5 genotypes (adults) were distinguished, but on av-

erage, fewer than 3 were distinguished in children and adults. Had there been 6 genotypes, there was nearly 85% probability of all 6 being detected. Upon oversampling, analysis of 20 isolates per individual demonstrated an average of fewer than 2 and 3 MS genotypes, respectively for children and adults. This finding is similar to (Emanuelsson and Thornqvist, 2000; Kozai et al., 1999; Li and Caufield, 1995; Tedjosasongko and Kozai, 2002) or less than the number of genotypes reported in other studies (*i.e*., less than 3 and 4, respectively children and adults) (Emanuelsson et al., 1998; Kohler et al., 2003; Kulkarni et al., 1989; Lembo et al., 2007; Liu et al., 2007; Marchant et al., 2001; Mattos-Graner et al., 2001; Mitchell et al., 2009; Napimoga et al., 2005; Redmo Emanuelsson et al., 2003). These differences may be due to the population of study or the method we used. In this regard, the population that participated in the study was from a high-caries-risk, 'poor access to dental care' group of African-Americans. Therefore, the representativeness of this study to other populations, including previous studies that focused on MS genotypes, may explain the differences observed.

The initial pilot data functioned as a model to estimate the power calculation to optimize effective numbers of isolates for individual. Using the data obtained and referring to the probability figure (Figure 1), we determined that 7-10 isolates was a reasonable number (and more practical than 20 isolates) for testing additional samples. In this regard, 7 isolates would provide 83% power of identifying up to 3 genotypes, while 10 isolates would have 78% power of identifying 4 genotypes; therefore, we continued our analysis with a group of samples from 35 children and 10 adult samples that had 7-10 isolates to observe how well genotypes were identified. The adult samples that had 7 to 10 isolates exhibited a genotype numbers similar to those of both child groups, with 83 to

95 % probability of detecting 3 genotypes (Table & Figure 1). Although the data were not statistically different for comparison of 20 and 7-10 isolates, for the adult sets of isolates, there were fewer genotypes identified with the 7-10 isolates as compared with the 20 isolate sampling. Therefore, the data suggest that sample sizes of 7-10 are sufficient number of isolates from younger (*i.e.*, the children in this study) individuals at a single time point to determine genetic diversity. However, further analysis may be indicated to better establish diversity in older individuals (*i.e.*, adults).

In conclusion, MS genotypes within individuals were efficiently analyzed by highly integrated rep-PCR and the microfluidics LabChip® assay to obtain representative genotypes from the oral microflora. By oversampling (20 isolates), the findings support the notion that representative genotypes are also detected by collecting 7-10 MS isolates *per* sample, especially in 5-6 year old children. These results provide information that will be used in future longitudinal studies as a database of MS genotypes and establishment of a library of genotypes in children and household family. Additionally, the methods using probability estimates provide a useful model for other microbiological studies involving genetic diversity of indigenous microorganism in nature.

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Table 1

Diversity of genotypes by subjects and sample sizes.

20 MS isolates of 12 children and 10 adults (N=22, total 440 isolates)

Number of genotypes	1	2	3	5	Mean genotypes
Number of children	5[a]	7[a]	0	0	1.6
% Subjects in group	42	58	0	0	
Number of adults	3[b]	2[b]	4[b]	1[b]	2.4
% Subjects in group	30	20	40	10	
Total subjects by genotypes	8	9	4	1	2.0
Overall % of subjects	36	36	23	5	

7-10 isolates of 35 children and 10 adults (N=45, total 473 isolates)

Number of genotypes	1	2	3	4	Mean genotypes
Number of children	22[a]	10[a]	1	2	1.5
% Subjects in group	63	29	3	6	
Number of adults	6[b]	3[b]	1[b]	0[b]	1.5
% Subjects in group	60	30	10	0	
Total subjects by genotypes	28	13	2	2	1.5
Overall % of subjects	62	29	4	4	

[a] No significant difference between 20 and 7-10 isolates of children groups by Fisher's exact test (p=0.3111).

[b] Statistically no differences between 20 and 7-10 isolates of adults groups by Fisher's exact test (p= 0.3698).

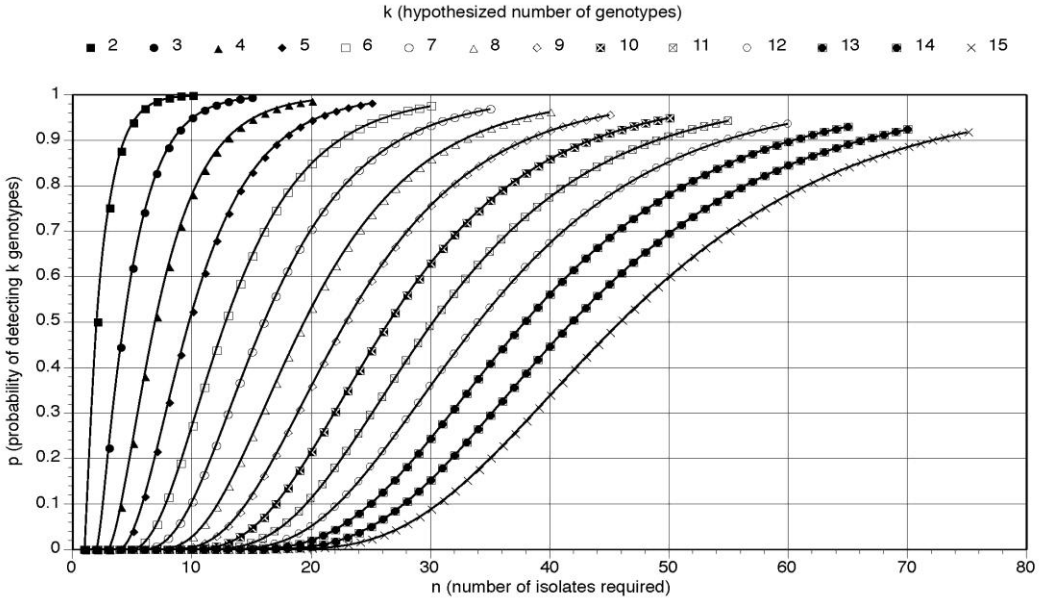

Figure 1. The number of isolates (n) estimated to be required to obtain a probability (p) of observing at least one of each hypothesized genotype (k) using the formula in the Power Calculations section of the Materials and Methods.

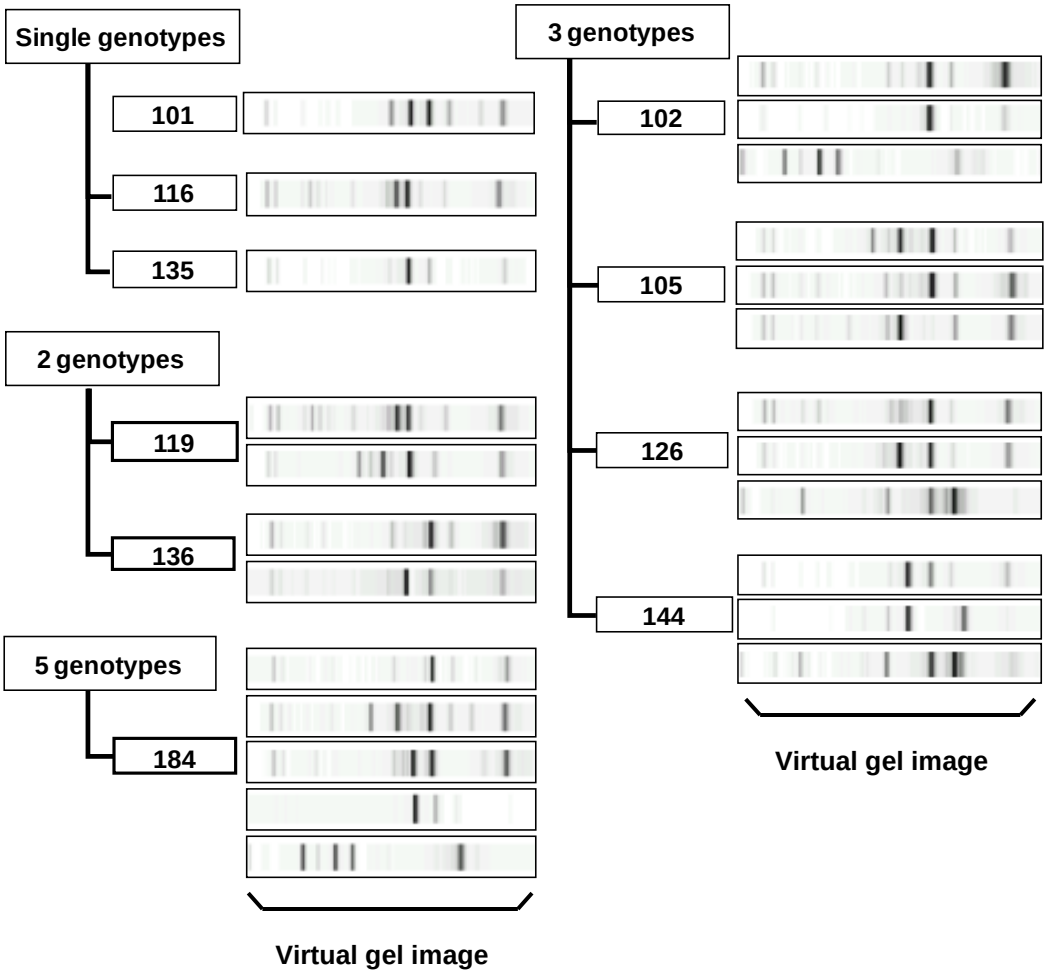

Figure 2. Representative rep-PCR results from 10 adults that had 20 isolates per individual analyzed. Virtual gel images represent DNA fingerprint patterns of MS isolates which were amplified with the automated rep-PCR system analyzed with DiversiLab software, version 3.3. The numbers identify individual that participated in this study.

CHARACTERIZATION OF *Streptococcus mutans* GENOTYPES IN HIGH CARIES RISK CHILDREN

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ABSTRACT

Streptococcus mutans is associated with the initiation of dental caries. A single *S. mutans* genotype has been shown to colonize primary teeth soon after eruption. More genotypes can be acquired with further dental eruption. This study investigates the diversity, commonality, and stability of *S. mutans* genotypes in children as permanent molars (PMs) erupt. Nineteen children ages 5 to 6 had plaque collected from primary or permanent molars at baseline (prior to eruption of at least one PM) and at six months. Seven to ten *S. mutans* isolates were obtained from each individual sample. DNA was extracted for repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) followed by microfluidics-based DNA amplicon fractionation with an Agilent 2100 Bioanalyzer. DNA fingerprints and dendrograms were constructed with the DiversiLab software (v3.3) using the Pearson Correlation Coefficient method and unweighted-pair group method with arithmetic mean (UPGMA) to interpret data. Among the nineteen children, a total of 621 isolates were analyzed and eleven genotypes were identified (average 33 isolates *per* child). The representative genotypes were labeled and stored as a web-based standard *S. mutans* library. Ten children had one genotype, eight had two genotypes, and one had three genotypes at baseline (average 1.5 genotypes: diversity). The most prevalent genotype was shared by six children, and the second most prevalent genotype was observed in three children at baseline (commonality). At six month follow up, eleven children's genotypes were identical (stability), and eight children acquired new or lost genotypes compared to baseline. These results initiated a longitudinal database for *S. mutans* geno-

typic distribution within and among children during dental development (while permanent teeth are erupting). *S. mutans* genotypes were summarized by diversity, commonality, and stability over time in this high caries risk group. Further analyses are planned for investigation of the relationship between *S. mutans* and dental caries.

Key words: *Streptococcus mutans,* genotype, rep-PCR, diversity, commonality, stability

INTRODUCTION

Dental caries has been known as a multifactorial disease (Keyes, 1968); therefore, it is difficult to predict individuals at risk. *Streptococcus mutans* has been identified as the primary etiological agent associated with the initiation of dental caries in humans. To understand the infectious nature of dental caries, associated with transmission of *S. mutans*, the concepts of diversity, commonality and stability were introduced. "Diversity" is the number of *S. mutans* genotypes found within an individual. "Commonality" is the *S. mutans* genotypes shared among individuals in the population. "Stability" is the persistence of *S. mutans* genotypes over time.

S. mutans strain identification traditionally utilized biotyping by characterizing phenotypic traits, *e.g.*, serotype (Bratthall and Kohler, 1976), bacteriocin production (Rogers, 1975) and biochemical testing (Facklam, 1977; Hamada and Slade, 1980) . Genetic analysis has now become an accepted approach for strain identification due to enhanced reliability and reproducibility (Whiley and Beighton, 1998). Interestingly, children usually carry fewer genotypes than adults, suggesting that additional strains may be acquired as one ages and that the "window of infectivity" (Caufield et al., 1993) may remain open indefinitely (Alaluusua *et al*., 1996; de Soet *et al*., 1998; Kozai *et al*., 1999). Also, children that manifest severe early childhood caries, or "bottle caries," have a greater diversity of *S. mutans* genotypes than caries-free children, indicating that frequent sugar consumption may exert a strong selective pressure for colonization with additional *S. mutans* genotypes (Alaluusua *et al*., 1996).

The purpose of this study was to determine the clonal diversity, commonality, and stability of *S. mutans* in the plaque of a cohort of school age children (high caries risk group) during a time when new permanent molar teeth are erupting. Genotypic characterization of *S. mutans* from this study will provide vital information about the association between *S. mutans* and dental caries for future studies.

MATERIALS AND METHODS

Population and information

Ninety-two children were enrolled in this IRB approved study with informed consent from their parents. Seventy-two out of ninety-two children participated in the follow up study. Nineteen of seventy-two children had samples collected from baseline and 6 months follow-up. Parents provided informed consent for their child's participation. Any children with all four first permanent molars erupted (any stage) were excluded from the study. An additional eligibility criterion was the absence of any severe medical conditions, especially those requiring long term antibiotic use prior to dental treatment. The children received a dental prophylaxis (rotary cup or toothbrush), preventive instructions, and a fluoride treatment.

Sample collection

At each visit, children had a pooled plaque sample collected from mesial, distal, buccal, lingual, and occlusal surfaces of primary second molar teeth and any permanent molars that were erupted at the baseline visit using a sterile toothpick. Samples were transferred to 1 mL of sterile reduced transport fluid (Syed and Loesche, 1972). Plaque

samples were transported to the laboratory on ice for processing within 24 hours of collection. Samples were collected again after 6 months with additional individual plaque samples collected from up to two newly erupting permanent first molars.

Isolation of *S. mutans*

The transported samples were gently mixed and sonicated for 30 seconds on ice at an amplitude of 50 (Vibra Cell, Sonics & Materials Inc., Newtown, CT, USA). Following dispersal in a 10^{-1} and 10^{-3} dilution using 0.05 M potassium phosphate buffer, each sample was plated in duplicate onto Mitis Salivarius agar (MS agar, Difco/ Becton Dickinson, Sparks, Maryland, USA) plates supplemented with 20% sucrose and 200 unit/liter of Bacitracin (Gold *et al*., 1973) with the Spiral Plater (Spiral System™ Inc, Cincinnati, OH, USA). Culture plates were incubated at 37°C in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% H_2) for 2 days (Lennette *et al*., 1974). *S. mutans* were identified by colony morphology and counted on the Gold's media. Seven to ten colonies *per* individual (Cheon *et al*., 2010) were collected and transferred for inoculation into Todd-Hewitt Broth (THB) (Beckton Dickinson, Sparks, Maryland, USA). Cultures were incubated for 24 hours under anaerobic conditions at 37°C. The samples were stored at $^{-}80^{o}$C for future processing in 20% glycerol. In this regard, frozen samples were plated to Todd- Hewitt Agar (THA) and grown anaerobically at 37ºC for 2 days. Isolated colonies ("isolates") were then inoculated into THB and incubated in an anaerobic atmosphere for 18-24 hours.

Extraction of DNA and rep-PCR

Cells obtained from the THB isolate cultures were processed by methods previously described to extract DNA (Cheon *et al*., 2010). Briefly, DNA was extracted with the Ultra Clean™ Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Quantitation of DNA was performed by a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) to control the quality of DNA (Cheon *et al*., 2010). All isolates were confirmed as *S. mutans* prior to genotyping by means of SYBR Green real time PCR according to a method described previously (Moser et al., 2010; Yoshida et al., 2003). Rep-PCR was performed with DiversiLab streptococcus kit (bioMerieux, Durham, NC, USA) and ABI9700 (Applied Biosystems, Foster City, CA, USA) according to the following parameters: initial denaturation of 94ºC for 2 min; 35 cycles of denaturation at 94ºC for 30 seconds; annealing at 50ºC for 30 seconds; extension at 70ºC for 90 seconds; and a final extension at 70ºC for 3 minutes.

DNA Chip application and DNA Fingerprints Analysis

The rep-PCR products were separated on microfluidics chips and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA.) with the DNA Chip kit (bioMerieux, Inc., Durham, NC, USA) (Bou *et al*., 2000; Healy *et al*., 2005). A molecular-weight ladder and Ingbritt C (*S. mutans* positive control) were run on each chip to assure consistency of results. A negative control was run on the first chip to verify absence of contamination for each rep-PCR batch of samples. Virtual gel images of each strain were generated by DiversiLab v 3.3 software (bioMerieux, Inc., Durham, NC, USA). The Pearson correlation coefficient method was used to determine distance matrices for simi-

larity calculations of DNA band intensity and location. The unweighted-pair group methodology with arithmetic mean (UPGMA) was utilized to create dendrograms, which provided a hierarchical cluster representation of similarities between samples and indicated strain level groupings of the *S. mutans*. The genotype distinction criteria of DNA fingerprint was followed by the methods previously described (Cheon *et al*., 2010; Moser *et al*., 2010); If two isolates differed by one major band or more than two minor bands, they were defined as distinct genotype. The generated reports were stored in DiversiLab's database with access via website and used for analysis as well as to construct a genotype library of *S. mutans* for future study.

RESULTS

A total of 621 isolates were analyzed from baseline and a 6 month follow-up of 19 children. To demonstrate genotypic diversity, the number of genotypes of *S. mutans* was evaluated within an individual and among a group. Individually, eighteen children had 1 or 2 genotypes and only one child had 3 different genotypes at baseline (Figure. 1). Similarly, seventeen children had 2 or less genotypes and two children had 3 genotypes at 6 month follow up (Table). As a group of 19 children, ten genotypes (G, i.e., G1, G6, G7, G9, G11, G12, G13, G14, G18, and GB) were defined at baseline and one more new genotype (G5) was added at 6 month collection of 19 children (Figure 2).

To evaluate commonality of genotypes, genotypic prevalence amongst children was summarized. The most prevalent genotype (G1) was shared by 6 children at both baseline and 6 months. The second-most prevalent genotype at baseline (G18) was shared by 3 children, while G9 was the second-most prevalent genotype at 6 months. Six geno-

types (G6, G7, G9, G13, G14, and GB) were not shared with anyone at baseline. A total of nine genotypes (G1, G6, G7, G9, G11, G12, G13, G18, and GB) were shared by at least two children at 6 month collection. G5 and G14 were unique genotypes of one of each child at 6 months (Figure 2).

Stability was measured with 3 different groups based on presence of number of genotypes at baseline, *i.e.*, single, two, and three genotypes at baseline. For the single genotype group (10 children), eight of ten children retained the same genotype from baseline to 6 month follow up. Two children, however, acquired a new genotype, yet still retained the predominant genotype from baseline at 6 month follow up. For the baseline two genotype group (8 children), three of eight children lost a "minor" (i.e., less than 2 of 7-10 isolates) genotype at 6 months; another three retained the same genotypes from baseline to 6 months; and two children acquired one new genotype at 6 months (maintaining the other two from baseline). For the three genotype individual (1 child), 2 minor genotypes were lost and the predominant genotype was retained at 6 months. Overall, the majority of children (17/19) retained their predominant genotype at the 6 month follow-up (Figure 3).

DISCUSSION

This study evaluated the diversity of *S. mutans* genotypes and found that the majority of children (94% at baseline and 89% at 6 months) had less than two genotypes. The mean number of genotypes of *S. mutans* at both collection periods was 1.5, which is consistent with our previous study of 35 children (Cheon et al., 2010). This result was supported by the other studies which showed that children have three or fewer genotypes

(de Soet et al., 1998; Emanuelsson et al., 1998; Emanuelsson and Thornqvist, 2000; Kozai et al., 1999; Li and Caufield, 1995). Since newly erupting permanent molar teeth occurred with some children between baseline and 6 month samples, the consistent mean genotypes of *S. mutans* with time may indicate that the eruption of permanent molar teeth has minimal effect on genotype diversity. Comparing to baseline, only one new genotype was introduced at 6 months, and this new genotype was found in only one child. This shows that the diversity of *S. mutans* genotype was stabilized early before eruption of permanent molars (Table). Other studies have demonstrated that *S. mutans* infections at an early age (mean age = 5 years old) are stable and consistent at least until permanent teeth erupt (Alaluusua *et al*., 1994; Emanuelsson and Thornqvist, 2000). Further additional longitudinal data will be needed to confirm our observations.

For determining commonality (Figure. 2), 68% of the children (13/19) at baseline and 89% of the children (17/19) at 6 months shared at least one genotype among a group. This result shows that the sharing pattern of *S. mutans* increased after follow-up. This implies the possibility of horizontal transmission along with vertical transmission. Recent studies suggest 31% paternal transmission (Kozai et al., 1999), and significant horizontal transmission from siblings and playmates (Liu et al., 2007; Mattos-Graner et al., 2001; Tedjosasongko and Kozai, 2002). Our study did not propose vertical transmission at this point, but further conclusions can be drawn when the children's profile is combined with adults' samples in a future study. Thus, our genotype analysis has provided insight into the nature of horizontal *S. mutans* transmission among children while vertical transmission between children and their family members, including adult caregivers is will follow.

The design of this study aimed to observe the stability of *S. mutans* that children are colonized over a six month period (Figure. 3), especially when new permanent molar teeth are erupting. Whether or not an initial "window of infectivity" (Caufield *et al*., 1993) exists, when new teeth erupt there is an opportunity to become colonized by *S. mutans* with a "new" genotype. Once a new genotype colonized primary teeth, no appreciable major genotypic shifts were observed associated with newly erupting permanent molar teeth. We found that the predominant genotype of each child was persistent until 6 months by most of children (17/19). The genotyping pattern of two children out of nineteen children showed differences from the other seventeen children. One child had 2 genotypes at baseline and retained the same 2 genotypes, but the proportion of the 2 genotypes were interchanged (Figure 3, child 150). The other child had 2 genotypes at baseline and acquired a new genotype resulting in having 3 genotypes at 6 months, and the new genotype appeared as the major genotype (Figure 3, child 149). This unique observation of child 150 and child 149 may lead to our further investigation to explain the susceptibility to dental caries associated with dmfs/DMFS. Thus, the findings of this study are important to determine relationships between the natural history of colonization and caries associated *S. mutans*.

In conclusion, a limited number of genotypes were identified within (and between) subjects, coupled with the finding that many genotypes were common and stable within and between individuals. This provides an important opportunity to provide supporting data to track potential sources of colonization with *S. mutans*. In this regard, this study is part of a larger study that will include similar analyses of *S. mutans* in household family members (*i.e*., mother, father, siblings, and other cohabitating individuals such as

aunts, uncles, cousins, and grandparents) of these children. One aspect of the study population is that 19 children were selected based on successful identification of *S. mutans* from baseline and 6 month plaque samples. Some children we sampled did not have cultivatable *S. mutans* from plaque at baseline or at 6 months. Nonetheless, these children are continued participants in the longitudinal study described and will be valuable to assess colonization with *S. mutans* over time, especially, as new permanent teeth erupt. Our future studies will focus on the relationship of colonization of genotypic characteristics with dental caries experience.

The rep-PCR method is a highly efficient and reproducible method of characterizing genetic features such as diversity, commonality, and stability of *S. mutans* by allowing the comparison of large quantities of data.

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Table

Number of genotypes by subject: Diversity of *S. mutans* genotypes among 19 children

Number of genotypes	1	2	3	4	Mean
Baseline (246 isolates)					
Number of subjects (%)	10 (53)	8 (42)	1 (5)	0	1.5
6 months (375 isolates)					
Number of subjects (%)	12 (63)	5 (26)	2 (11)	0	1.5

%: percent of subjects that had given number of genotypes are shown in parenthesis

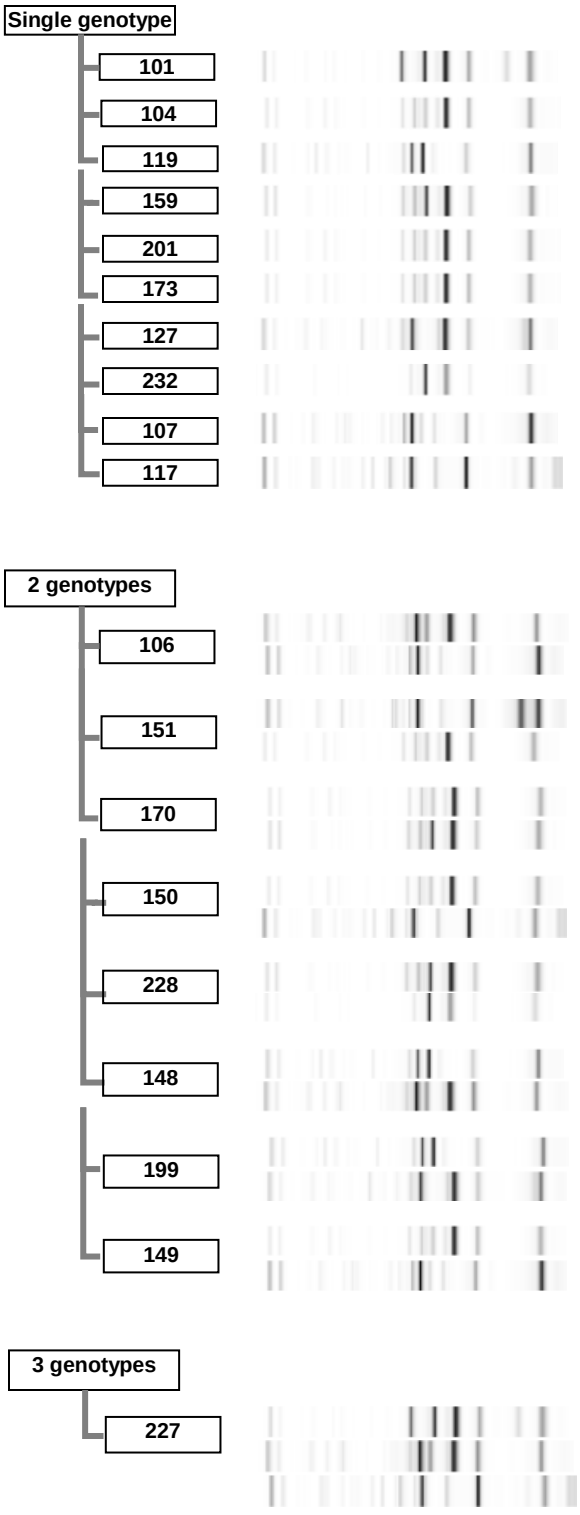

Figure 1. DNA fingerprint: Three different groups of *S. mutans* genotypes (Baseline only, 246 isolates from 19 children)

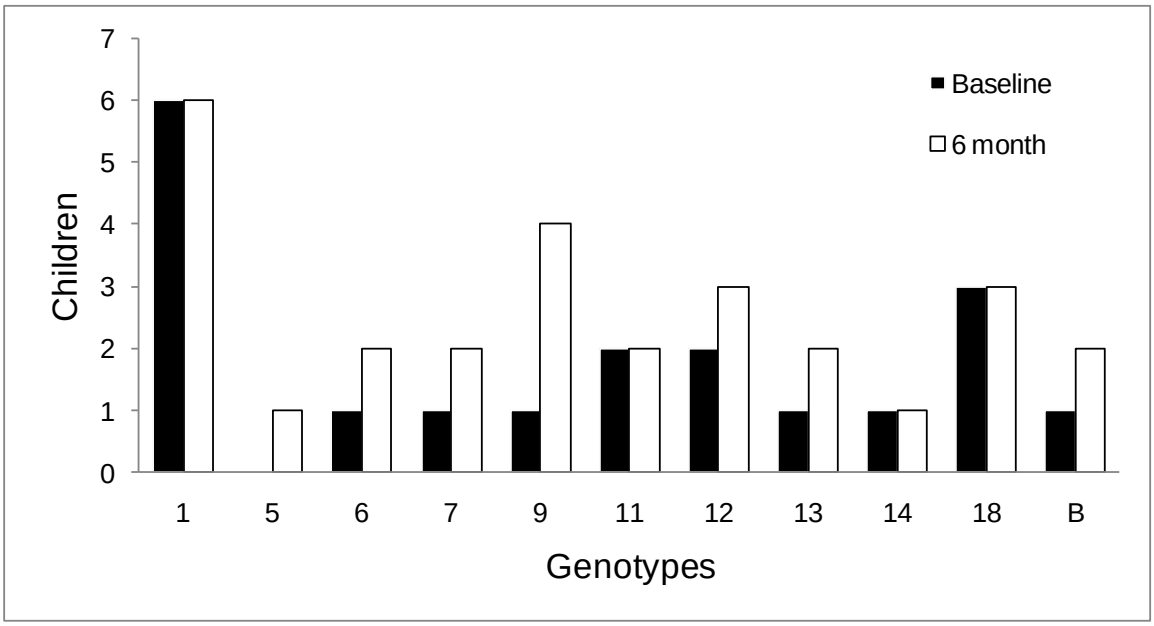

Figure 2. Shared genotypes: Commonality of *S. mutans* genotypes (Baseline and 6 month, 621 isolates from 19 children)

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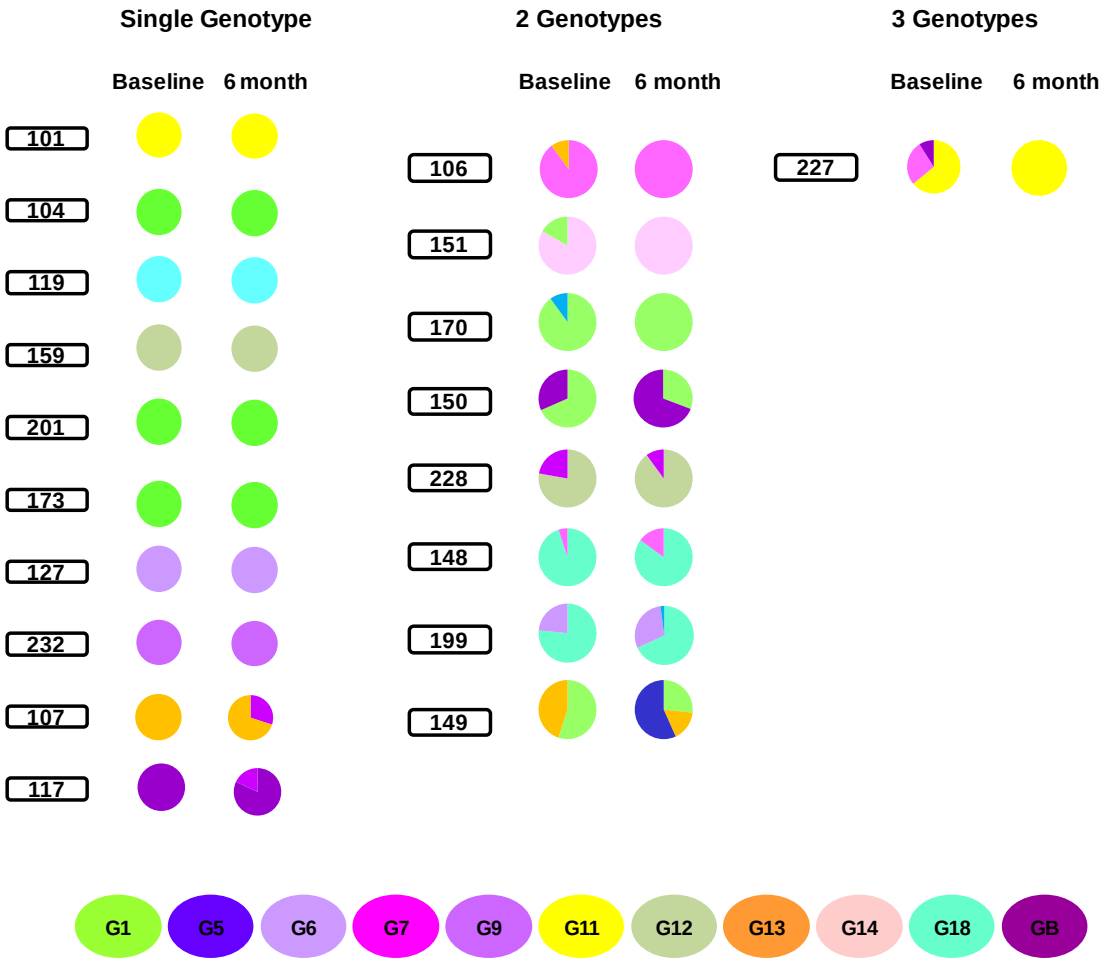

Figure 3. Maintained genotypes: Stability of *S. mutans* genotype
(Baseline and 6 month, 621 isolates from 19 children)
The numerical ID indicates the 19 children and the pie diagram represents the proportion of each genotype from 7-10 isolates analyzed from each plaque sample

GENERAL CONCLUSIONS

Our study indicated that the most predominant genotypes can be detected by sampling 7-10 isolates per individual. Even if a few minor genotypes were detected, those genotypes comprised less than 0.1 % of the total sample size in an individual. The characteristics of MS genotypes can be analyzed by three main categories: diversity, commonality, and stability. Diversity of MS genotypes was found to be limited. Greater than 90% of children had one or two genotypes with a mean of 1.5 genotypes overall. Furthermore, all children had one predominant genotype regardless of collection period**.** Commonality of MS genotypes was observed by 68% and 89% of children, respectively at baseline and 6 months collection**.** Stability of MS genotypes was 100% during follow up. Although some children lost or acquired MS genotypes, they all maintained at least one baseline genotype through the 6 months of this study.

These findings will be a strong foundation for future 24 month longitudinal comparisons within and between children and family members. Moreover, these MS genetic findings, when combined with analysis of other factors, (*e.g.*, diet history, dmfs/DMFS, and salivary anti-MS antibody activity) will provide a better understanding of the infectious nature of dental caries in high risk groups of children.

Highly integrated rep-PCR with the Microfluidics LabChip® assay is efficient for high throughput screening of MS genotypes within individuals and useful for continual database construction.

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APPENDIX

IRB APPROVAL

Project Revision/Amendment Form

(Rev. 4/7/2004)

Link: Project Revision/Amendment Form

Federal regulations require IRB approval before implementing proposed changes.
Please complete this form and attach the changed research documents. Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.)

Principal Investigator: *Noel K. Childers, DDS* Date: *July 20, 2007*

Contact: *Stephanie McLean* Phone #: *6-6628* Fax #: *4-7013* E-mail: *smclean@uab.edu*

Campus Address: *SDB 304B*

Study/Protocol Title: *Epidemiology of Dental Caries and Immunity in Children (Alabama)*

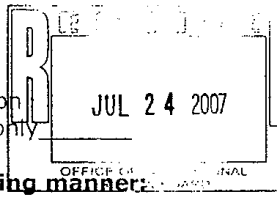
IRB Protocol #: *F060328001*

Current Status of Project: (check only one)

☒ Currently in Progress (# participants entered: *94*

☐ Study has not yet begun (no participants entered)

☐ Closed to participant enrollment (remains active); # participants on therapy/intervention_____; # participants in long-term follow-up only_____



This submission changes the status of this study in the following manner: (check all that apply)

☐ Protocol Revision
☐ Protocol Amendment
☐ Study Closed to participant entry
☐ Study Terminated
☐ Revised Consent Form
☐ Addendum (new) consent form
☐ Enrollment temporarily suspended by sponsor
☒ Other, (specify) *Addition of Anrika McConnico and Kyounga Cheon to the project, their Initial Human Subjects Training verifications are attached*

1. Briefly describe, and explain the reason for, the revision or amendment. Include a copy of supportive documents with changes highlighted. Please highlight changes/revisions/additions to the consent form, protocol, research questionnaire, etc.
We are adding Anrika McConnico and Kyounga Cheon to the list of study personnel.

2. Does this revision/amendment revise or add a genetic or storage of samples component? ☐ Yes ☒ No
If yes, please see the Guidebook to assist you in revising or preparing your submission documents or call the IRB office at 4-3789.

3. Does the change affect subject participation (e.g. procedures, risks, costs, etc.)? ☐ Yes ☒ No

4. Does the change affect the consent document? ☐ Yes ☒ No
If yes, briefly discuss the changes.

Include the revised consent form with the changes highlighted.

Will any participants need to be reconsented as a result of the changes? ☐ Yes ☒ No
If yes, when will participants be reconsented?_____

OMB No. 0990-0263
Approved for use through 1/31/2012

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule.

Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [X] CONTINUATION [ ] EXEMPTION	[ ] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	
4. Title of Application or Activity Epidemiology of Dental Caries and Immunity in Children (Alabama)		5. Name of Principal Investigator, Program Director, Fellow, or Other CHILDERS, NOEL

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 10/26/2010 IRB Registration No. IRB00000726

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [X] Full IRB Review on (date of IRB meeting) 11/11/2009 or [ ] Expedited Review on (date) _____
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments	Title F060328001
Protocol subject to Annual continuing review.	Epidemiology of Dental Caries and Immunity in Children (Alabama)

IRB Approval Issued: 12/08/09

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: smoore@uab.edu	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
14. Name of Official Ferdinand Urthaler, M.D.	15. Title Chairman, IRB
16. Signature Ferdinand Urthaler, MD	17. Date 12/08/09

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Signature of Principal Investigator Karl K Chilcote Date 7/29/07

FOR IRB USE ONLY

dola 3-7-07

APPROVED
Denise Ball 07-24-07
IRB REVIEWER

Revised 04-07-04