REAL-TIME QUANTITATIVE PCR FOR EVALUATION OF MUTANS STREPTOCOCCI AND DENTAL CARIES

by

CHANIKA MANMONTRI

NOEL K CHILDERS, COMMITTEE CHAIR
AMJAD JAVED
GARY R. CUTTER
JOHN D. RUBY

A THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Master of Science

BIRMINGHAM, ALABAMA
2012
REAL-TIME QUANTITATIVE PCR FOR EVALUATION OF MUTANS STREPTOCOCCI AND DENTAL CARIES

CHANIKA MANMONTRI

DENTISTRY

ABSTRACT

Mutans streptococci (MS), e.g., *Streptococcus mutans* (*Sm*) and *Streptococcus sobrinus* (*Ss*), are associated with dental caries. Quantification of MS has been shown to predict caries risk. Traditional culture methods are problematic, especially because consistently maintaining the viability of bacteria is difficult. A new technology using PCR has the potential to streamline and improve MS quantification compared to traditional culture methods.

**Purpose:** To use real-time quantitative PCR (qPCR) and standard plate counting (SPC) for detection and quantification of levels of *Sm*, *Ss* and total streptococci (TS) in clinical samples from high-caries-risk children (no regular access to dental care) and to evaluate the correlation of these results to caries prevalence.

**Methods:** Saliva and plaque samples were collected from school-aged children for SYBR Green based qPCR using primers specific for *Sm*, *Ss* and TS from DNA extracted. UA159 genomic DNA was used as standard from which “copy number” (CN/ml) of MS and TS was computed. For comparison, samples were also processed and quantified for “colony forming unit” (CFU/ml) of MS and TS using SPC. Calibrated
examiners performed oral examinations (DMFT/DMFS/dmft/dmfs). Pearson’s analysis and T-test assessed correlations between level of \( Sm \) and ratio of \( Sm \) to TS to caries using SAS V9.2.

**Results:** Fifty-eight subjects (mean age=9.8 years, DMFS/dmfs=7.0) had samples collected. By qPCR \( Sm \) was detected in 98.1% of samples while SPC was 72.9%. \( Ss \) was only found in 4 qPCR samples (none by SPC). Mean qPCR CN/ml of samples were: \( Sm \) (2.54x10^6), \( Ss \) (5.27x10^5) and TS (2.34x10^9). Mean SPC CFU/ml of samples were: \( Sm \) (9.4x10^5), \( Ss \) (0) and TS (1.0x10^7). Significant correlations were found between level of \( Sm \) and ratio of \( Sm/TS \) from oral samples to prevalence of dental caries.

**Conclusion:** The levels of \( Sm \) and ratio of \( Sm/TS \) by qPCR and SPC were correlated to dental caries. Correlations between mean levels of \( Sm \) and ratio of \( Sm/TS \) to caries experience from qPCR are comparable to SPC. Given the similar correlations found, the more streamlined method of qPCR is a suitable outcome measure in epidemiological studies.

**Keywords:** real-time quantitative PCR, \( S. mutans \), \( S. sobrinus \), dental caries
DEDICATION

This thesis is dedicated to the love and memory of my father, Dr. Anon Manmontri. The first and best teacher of my life, he guided me with advices, supported me in every way, and inspired me into the academic career. I always keep him close to my heart thus he remains with me in all steps of my life.

To my family; my mother, Jumras Manmontri; my sisters, Boripont and Nawaporn Manmontri; my boyfriend, Wittawat Rawiyotai: thank you for your unceasing supports and encouragements. Being away from home was not easy but because of your love, care and trust in me that made me passed through the challenging years of staying and studying here.
ACKNOWLEDGEMENTS

I would like to thank …

My research mentor, Dr. Childers: for guiding me throughout my residency and my graduate study. Your dedication towards work and research inspires me in achieving the best for my academic career.

My research committee, Dr. Ruby, Dr. Cutter, Dr. Javed: for providing generous supports and advices for my Master’s degree project and thesis.

Dr. Cheon, Dr. Hsu, Dr. Mahtani: for laboratory training and providing advices for my project and my graduate study.

All the clinical and laboratory participants of this study: Ms Stephanie McLean, Ms Tonya Wiley, Ms Stephanie Momeni, Dr Steve Mitchell, Dr Sonia Makhija, Dr Rosalyn Bassett, Ms Mary Slater, Ms Frances Jackson, and the pediatric dental residents of the UAB School of Dentistry: for diligences and participations in this ongoing project at Uniontown.

Supports from my home university, Chiang Mai University (CMU), in Thailand and from University of Alabama at Birmingham (UAB): I am grateful to receive upholding supports from CMU Academic Staff Continuing Education in PhD and Equivalent Scholarship, Dr. Britta Rahemtulla Endowed Scholarship Fund Award for Achievement in Research and a scholarship from the UAB Graduate School Fellowship Program, School of Dentistry, UAB. Besides, this project was also supported by a Research Grant DE016684 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REAL-TIME QUANTITATIVE PCR FOR EVALUATION OF MUTANS STREPTOCOCCI AND DENTAL CARIES</td>
<td>12</td>
</tr>
<tr>
<td>GENERAL CONCLUSIONS</td>
<td>43</td>
</tr>
<tr>
<td>GENERAL LIST OF REFERENCES</td>
<td>47</td>
</tr>
<tr>
<td>APPENDIX: IRB APPROVAL FORMS</td>
<td>51</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Detection of <em>Sm</em> and <em>Ss</em> from oral samples by qPCR and SPC</td>
</tr>
<tr>
<td>2</td>
<td>Quantification of <em>Sm</em> and ratios of <em>Sm/TS</em> from oral samples by qPCR and SPC and correlation to caries index</td>
</tr>
<tr>
<td>3</td>
<td>Quantification of <em>Sm</em> and ratios of <em>Sm/TS</em> from oral samples by qPCR and SPC and correlation to active caries</td>
</tr>
<tr>
<td>4</td>
<td>Quantification of <em>Sm</em> and ratios of <em>Sm/TS</em> from oral samples by qPCR and SPC and T-test analysis to caries status</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>2a</td>
<td>41</td>
</tr>
<tr>
<td>2b</td>
<td>42</td>
</tr>
</tbody>
</table>

1. Standard curve of a ten-fold dilution series of known template concentrations of *S. mutans* UA 159.
2a. Melting curve chart for *S. mutans* and total streptococci.
2b. Melting curve chart for *S. mutans* and *S. sobrinus*. 
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CN</td>
<td>copy number</td>
</tr>
<tr>
<td>Cq</td>
<td>quantification cycle</td>
</tr>
<tr>
<td>DMFS</td>
<td>decay, missing, filled surface score in permanent teeth</td>
</tr>
<tr>
<td>dmfs</td>
<td>decay, missing, filled surface score in primary teeth</td>
</tr>
<tr>
<td>DMFT</td>
<td>decay, missing, filled tooth score in permanent teeth</td>
</tr>
<tr>
<td>dmft</td>
<td>decay, missing, filled tooth score in primary teeth</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>gtf</td>
<td>glucosyltransferases</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MS</td>
<td>mutans streptococci</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RTF</td>
<td>reduced transport fluid</td>
</tr>
<tr>
<td>Sm</td>
<td><em>Streptococcus mutans</em></td>
</tr>
<tr>
<td>SPC</td>
<td>standard plate counting</td>
</tr>
<tr>
<td>Ss</td>
<td><em>Streptococcus sobrinus</em></td>
</tr>
<tr>
<td>TE buffer</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TS</td>
<td>total streptococci</td>
</tr>
<tr>
<td>UAB</td>
<td>the University of Alabama at Birmingham</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Dental caries is a major infectious disease that affects most populations around the world, especially children. This disease can adversely impact their weight, growth, and well-being. Although preventable, caries has found to be one of the most common childhood diseases in the United States. In 2000, the Surgeon General’s report on Oral Health revealed that caries is more than 5 times as common as asthma and 7 times as common as hay fever reported in children aged 5 to 17 years old (1). Surveillance for dental caries, dental sealants, tooth retention, edentulism, and enamel fluorosis by the National Health and Nutrition Examination Survey (NHANES) during 1988-1994 and during 1999-2002 reported that despite improvement of caries prevalence in permanent dentition, the caries prevalence in primary dentition was stable and still a major concern (2). It was found that 40% of children aged 2 to 11 years during 1988-1994 had dental caries in primary teeth. The consecutive survey in 1999-2002 reported that there is no change in caries status in this age group; 41% of the children had dental caries in primary teeth (2). Moreover, dental caries is not only an epidemic health problem in USA: Oral health disparities among US population are also another major problem. More than one-third (36.8%) of children with low socioeconomic status at aged 2 to 9 years old had untreated carious teeth, compared to 17.3% of children with high socioeconomic status. Nevertheless, in the group of low socioeconomic status children, there are also disparities among ethnicity. Hispanic American children aged 2 to 9 years old have the highest
number of carious-affected primary teeth (a mean of 2.4 decayed or filled teeth: dft) compared to non-Hispanic African Americans (mean 1.5) and non-Hispanic Caucasians (mean 1.9) children (1). Consequently, caries risk assessment has been proposed as an essential tool for targeting children at risk to provide specific treatment plan and caries preventive plan for individual dental patient.

**Dental Caries and Mutans Streptococci**

Although infectious, dental caries is multifactorial (i.e., dietobacteria) making it a complex chronic disease. Data suggest that mutans streptococci (MS) play an important role in the initiation and development of dental caries (3, 4). Human MS species are *Streptococcus mutans* and *Streptococcus sobrinus*. These bacterial species have been reported to be detected in oral samples, such as plaque, saliva and tongue scrapings (5, 6). The observance of a high proportion of MS to total cultivable bacteria in dental plaque is an indicator associated with carious lesion initiation and progression. A high proportion of MS is consistently found in plaque of cavitated lesions. On the contrary, plaque of non-carious tooth in caries-active subjects or in plaque of caries-free subjects contained much lower proportions of MS to total cultivable bacteria (3, 7).

Additionally, many studies suggested that MS are a major pathogen of early childhood caries (ECC) by showing significant correlation between detection of MS in saliva and plaque samples to severity of ECC (8-14). ECC is a term adopted by the American Academy of Pediatric Dentistry that refers to a severe form of dental caries in childhood which has various adverse consequences effecting child’s health and well-
being. The definition of ECC is “the presence of 1 or more decayed (noncavitated or
cavitated lesions), missing (due to caries), or filled tooth surfaces in any primary tooth in
a child under the age of 6”(15). A systematic review by Thenisch et al. in 2006 concluded
that presence of MS in plaque and saliva of caries free children age 2-5 years is
significantly associated with increased risk of developing ECC. They found that the
pooled relative risk for developing ECC was 3.85 when children harbored MS in plaque
and was 2.11 when MS was harbored in saliva (16).

In summary, evidence supporting the statement that MS is a major pathogen of dental
caries (17, 18) consist of the following:

- MS are frequently isolated from carious lesions,
- germ free rats do not develop caries when fed with high sucrose diet unless MS is
  introduced to oral cavity,
- high acid-production (acidogenic) and acid-tolerance (aciduric) properties of MS
  contribute to survival in the dynamic environment of oral cavity,
- surface antigens I/II and water-insoluble glucan produced by MS enhance
  adhesion of MS and other bacteria to the tooth surface.

**Streptococcus mutans and Dental Caries**

*S. mutans* is found to be the most common streptococcal species isolated from
human oral cavity (5, 6). It accounted for 74% to 100% of the human isolates of MS from
various populations (5). Studies show that the level of *S. mutans* from clinical samples,
such as dental plaque and saliva, was highly associated with dental caries prevalence (10,
11, 13, 19, 20). A study of the microbiota of young children from tooth and tongue
samples by Tanner et al. in 2002 reported that *S. mutans* was often found in young children with caries experience. It was the most frequently detected species in 6 to 36 months old children when analyzed with checkerboard DNA probe hybridization method. Moreover, detection of *S. mutans* was highest in samples from children with more than two carious teeth (21). A study in Korean preschool children age less than 71 months old using real-time quantitative polymerase chain reaction (qPCR) to detect and quantify *S. mutans* in plaque samples also confirmed significant correlation of mean level of *S. mutans* to caries severity (*r*=0.211, *p*<0.05) (11). Similarly, a longitudinal study using standard bacterial culture for MS detection in Japanese children (2.5 years old) revealed that children that had MS colonized in plaque at the beginning of study had significantly higher dft score after a 2 year period than those who were negative for MS colonization (13).

A cross-sectional study using conventional PCR for MS detection in preschool Mexican children age 3 to 6 years old supported the concept that *S. mutans* is involved in the initiation of dental caries. However, *S. mutans* were detected in most children whether they were caries-active (75%) or caries-free (55%) without significant difference (*p*>0.05) between groups (10). Interestingly, when both *S. mutans* and *S. sobrinus* were detected in saliva, the detection rate was significantly higher in caries-active children (42.5%) than in caries-free (17.5%) children (*p*<0.05) (10). Nurelhuda et al. also found that level of *S. mutans* in saliva detected by qPCR for Sudanese children age 12 year old with caries activity were not significantly different from the children with no caries activity (*p*=0.96). However, the levels of *S. sobrinus* were significantly correlated to
presence of caries activity \((p=0.008)\) (14). These studies suggested that \(S. \textit{sobrinus}\) influences in severity of dental caries (10, 14).

\textit{Streptococcus sobrinus and Dental Caries}

As indicated in the previous section, some studies found that \(S. \textit{mutans}\) was not the only MS associated with dental caries. \(S. \textit{sobrinus}\) was also a common streptococcal species isolated from human oral cavity. In this regard, it was detected in 0\% to 53\% of the human isolates of MS of samples from various populations (5). The detection rate of \(S. \textit{sobrinus}\) may be related to the caries status of children. A cross-sectional study using conventional PCR for MS detection in preschool Mexican children age 3 to 6 years old found that \(S. \textit{sobrinus}\) in saliva was closely associated to caries prevalence. The study showed that the proportion of \(S. \textit{sobrinus}\) detection was significantly higher in caries-active children (52.5\%) than in caries-free (20\%) children \((p<0.05)\) (10). A study in Korean preschool children age less than 71 months old using qPCR to detect and quantify \(S. \textit{mutans}\) in plaque samples also confirmed significant correlation of mean level of \(S. \textit{sobrinus}\) to caries severity \((r=0.437, p<0.01)\) (11).

Not only was the detection of \(S. \textit{sobrinus}\) associated with caries prevalence, but also the level in oral samples was strongly associated with caries prevalence (10, 11). Loyola-Rodriguez \textit{et al.} in 2008 suggested that \(S. \textit{sobrinus}\) was more associated with active dental caries and caries experience because it could be detected almost twice as much in caries-active children. They found that the mean \(S. \textit{sobrinus}\) CFU in caries-active children \((6.5\times10^4)\) was significantly higher than in caries-free children \((7.5\times10^3)\).
They also found that the higher index of decayed, missing and filled surfaces index (dmfs), the higher level of *S. sobrinus* detected (10).

**S. mutans with S. sobrinus and dental caries versus S. mutans alone**

Some studies have reported that caries severity had higher correlation to detected MS when *S. mutans* presented with *S. sobrinus* than when *S. mutans* presented alone. A cross-sectional and 1-year longitudinal study in Japanese preschool children age 3 to 5 years old also showed that the incidence of caries was significantly higher when children harbored both *S. mutans* and *S. sobrinus* in plaque when compared to those who were positive for *S. mutans* alone (8, 9). A 1-year longitudinal study in Japanese children also supported this finding with children that harbored both *S. mutans* and *S. sobrinus*. Children with both species were found to have a slight tendency for higher levels of caries prevalence when compared to children who harbored *S. mutans* alone (12).

**Ratio of MS and Dental Caries**

Other studies have shown high correlation between ratio of *S. mutans* to total bacteria in clinical samples and prevalence of dental caries as well as ratio of *S. sobrinus* to *S. mutans* and prevalence of dental caries (8, 9, 11, 14, 19, 20). Choi *et al.* in 2009 found a significant correlation of caries severity to ratio of *S. mutans* to total streptococci (r=0.301, \(p<0.05\)), ratio of *S. sobrinus* to total streptococci (r=0.344, \(p<0.01\)), and ratio of *S. sobrinus* to *S. mutans* (r=0.7488, \(p<0.05\)) (11). Hata *et al.* compared ratio of *S. mutans* to total streptococci from decayed maxillary primary central incisors of Japanese preschool children age 3-6 year using qPCR with the ratio from sound incisors. They found that the ratio from children that have carious incisors were three or four times as
high as those with sound incisors \( (p<0.01) \) (19). Loyola-Rodriguez et al. in 2008 presented that the mean of dmfs for the caries-active children group was positively correlated with ratio of \( S. \ mutans \) to \( S. \ sobrinus \) \( (r=0.3978, \ p=0.0003) \) (10). Similarly, Nurelhuda et al. also found that ratio of \( S. \ mutans \) to \( S. \ sobrinus \) in saliva for Sudanese children age 12 year old were significantly correlated to presence of caries activity \( (r=0.23, \ p=0.007) \) (14) Consequently, quantifying MS and total streptococci (TS) in oral cavity is valuable information in defining individual caries risk (7, 22).

Detection of MS in Oral Cavity

Detection of MS in the oral cavity can be important information to help predict caries risk (8, 9, 11, 14, 19, 23). It has been reported that the earlier the MS colonize children, the higher the caries prevalence (13, 22, 24, 25). Several methods have been used to detect MS from oral samples, for example; direct microscopy, cultivation, enzyme test, monoclonal antibodies (mAbs), enzyme-linked immunosorbent assays (ELISA), species specific DNA probes such as conventional end-point PCR (8, 9, 11, 14, 19). Most of these methods are time-consuming, laborious, and relatively unspecific (11).

Bacterial culture on specific media is a routine method in detection and quantification of MS although it does not reveal diversity of bacterial biofilm communities. Traditionally, Mitis-salivarius agar and Mitis salivarius-bacitracin agar has been used for culturing MS. By this method \( S. \ mutans \) and \( S. \ sobrinus \) can be distinguished by differences of colony morphology (12, 19, 26). \( S. \ mutans \) colonies are multiberry-shaped rough type colony which grows into the agar whereas \( S. \ sobrinus \)
colonies have smooth surface and are usually surround by extracellular polysaccharides. MS can also be classified as *S. mutans* and *S. sobrinus* using biological properties. *S. mutans* ferments sorbitol, mannitol, raffinose and melibiose but cannot agglutinate dextran. *S. sobrinus* ferments mannitol, but not raffinose or melibiose, but can agglutinate dextran (12). This method of detecting and classifying MS is subjective, time-consuming, labor-intensive, and impractical in field epidemiological studies (9, 19, 27-29).

**Real-time Quantitative Polymerase Chain Reaction (qPCR)**

PCR is also used for detection and quantification of MS. The advantages of PCR are high sensitivity and specificity in detection of oral bacteria of interest (30, 31). In order to quantify bacterial species in biological samples, qPCR was introduced as a novel method for rapid detection and quantification of bacterial species (32). Using sequence-specific primers of a particular DNA sequence of genes of interest, bacterial numbers can be quantified from the amplification of double stranded DNA products. While performing PCR cycles, the amplified DNA products are bound by fluorescent DNA dyes, allowing determination of relative number of copies of DNA.

The major advantage of qPCR is that it overcomes the inability of detecting non-viable bacteria in culture methods (11, 29, 33). Consequently, transportation and storage of clinical samples will not be a critical concern as in the method using conventional bacterial culture (29). Therefore, easy sample handling can be a benefit of qPCR for an epidemiologic study with a large-scale population, for instance, samples can be stored on ice or frozen immediately and analyzed at a later date (11, 29). Also, based on primer design, qPCR is highly specific and sensitive. This technique has been shown to be more
sensitive than conventional bacterial culture in detection of targeted bacterial species (8, 9, 30, 34-36). Psoter et al. reported that qPCR is more sensitive than conventional PCR and suggested that the technique is suitable for field oral epidemiologic studies (27). The assay can be used to detect low numbers of bacterial species of interest with a detection limit as low as 25-100 cells (8, 9, 31, 34-36). The specificity of qPCR is accomplished using designed primer sets that are specific to gene sequence of bacterial species of interest. This results in a great benefit of qPCR in obtaining precise distinguishability between S. mutans and S. sobrinus for accurate caries risk prediction and effective dental caries preventive plan (9).

Real-time quantitative PCR assay also allows relative or absolute quantification of bacteria of interest including species that cannot be cultured or are difficult to culture in vitro. The advantage of qPCR over conventional PCR or end-point PCR is that this assay does not require post-PCR manipulation of PCR products, which results in chance reduction of carryover-contamination of the products (11, 33). Also, qPCR requires less laboratory work and time to obtain results from various types of samples (37).

Several studies support the use of PCR and qPCR for detection and quantification of MS. Okada et al. used PCR to study intra-oral distribution of MS in children in a cross-sectional study in 2002 and longitudinal study in 2005 and suggested that PCR is suitable and effective in MS detection (8, 9). Loyola-Rodriguez et al. suggested that PCR is comparable to conventional bacterial culture on MS agar and MSB agar as a tool in detection and identification of MS (10). To date, there are several studies that have used qPCR to identify MS from oral samples, such as plaque and saliva samples. These studies also investigated the correlation of MS detection to dental status, i.e., caries status; decay,
missing, filling index of primary or permanent tooth (dmf or DMF), or caries activity; caries-free and caries-active status (8, 9, 11, 13, 14, 19, 20).

Studies Using qPCR for Detection and Quantification of MS and Correlation to Dental Caries

Several studies using qPCR for detection and quantification of MS from oral samples reported a high association between MS findings to caries prevalence (11, 13, 14, 19, 20, 27). The samples from these studies were collected from populations in Asia (11, 13, 19, 20), Africa (14) and Haiti (27). None of the studies were from North America. According to a longitudinal epidemiological study from high-caries-risk children in Perry County, Alabama using bacterial culture to detect and quantify MS from clinical samples, S. sobrinus were rarely detected in the population in this study (unpublished data). This preliminary finding corresponds to previous review by Loesche in 1986 (5) that the distribution frequency of MS vary among populations. However, due to limitation of bacterial culture in detecting and quantifying MS, qPCR is a potentially useful tool in this research study due to its higher sensitivity to validate the finding from bacterial culture. Therefore, the purpose of the study to follow is to compare the use of qPCR for quantification of level of S. mutans, S. sobrinus and total streptococci in clinical samples to bacterial culture and to study the correlation of the bacterial levels and ratios to caries prevalence.
Objectives

1. To study the correlation between the level of *S. mutans* and *S. sobrinus* in clinical samples and caries prevalence by qPCR and SPC.

2. To study the correlation between the ratio of *S. mutans* to total streptococci (*Sm/TS*) in clinical samples and caries prevalence by qPCR and SPC.

3. To study the correlation between the ratio of *S. mutans* to *S. sobrinus* (*Sm/Ss*) in oral samples to caries prevalence by qPCR and SPC.
REAL-TIME QUANTITATIVE PCR FOR EVALUATION OF MUTANS STREPTOCOCCI AND DENTAL CARIES

CHANlKA MANMONTRI, JOHN D. RUBY, GARY R. CUTTER, HOWARD W. WIENER, ROBERT C. OSGOOD, STEPHANIE S. MOMENI, TONYA WILEY, NOEL K. CHILDERs

In preparation for Pediatric Dentistry, a journal of the American Academy of Pediatric Dentistry

Format adapted for thesis
ABSTRACT

Mutans streptococci (MS), e.g., Streptococcus mutans (Sm) and Streptococcus sobrinus (Ss), are associated with dental caries. Quantification of MS has been shown to predict caries risk. A new technology using PCR may streamline quantification compared to traditional culture methods.

**Purpose:** To use real-time quantitative PCR (qPCR) and standard plate counting (SPC) for detection and quantification of levels of Sm, Ss and total streptococci (TS) in clinical samples from high-caries-risk children with no regular access to dental care and to study correlation of these bacterial levels and ratios to caries prevalence.

**Methods:** Saliva and plaque samples were collected from school-aged children for SYBR Green based qPCR using primers specific for Sm, Ss and TS from DNA extracted compared to SPC. UA159 genomic DNA was used as standard from which “copy number” (CN/ml) of MS and TS was computed. Samples were also processed and quantified for “colony forming unit” (CFU/ml) of MS and TS using traditional plate culture. Calibrated examiners performed oral examinations (DMFT/DMFS/dmft/dmfs). Pearson’s analysis and T-test assessed correlations between level of Sm and ratio of Sm to TS to caries using SAS V9.2.

**Results:** Fifty-eight subjects (mean age=9.8 years, DMFS/dmfs=7.0) had samples collected. By qPCR Sm was detected in 98.1% of samples while SPC was 72.9%. Ss was
only found in 4 qPCR samples. Mean qPCR CN/ml of samples were: Sm (2.54x10^6), Ss (5.27x10^2) and TS (2.34x10^9). Mean SPC CFU/ml of samples were: Sm (0.94x10^6), Ss (0) and TS (0.01x10^9). Significant correlations were found between level of Sm and ratio of Sm/TS from oral samples to prevalence of dental caries.

**Conclusion:** levels of Sm and ratio of Sm/TS by qPCR and SPC were correlated to dental caries. Correlations between mean levels of Sm and ratio of Sm/TS to caries experience from qPCR are comparable to SPC. Given the similar correlations found, the more streamlined method of qPCR is a suitable outcome measure in epidemiological studies.

Keywords: real-time quantitative PCR, *S. mutans*, *S. sobrinus*, dental caries
INTRODUCTION

Dental caries is a major infectious disease that affects most populations around the world, especially children. This disease can adversely impact children’s weight, growth, and well-being. Although preventable, caries has been found to be one of the most common childhood diseases in the United States. In 2000, the Surgeon General’s report on Oral Health revealed that caries is more than 5 times as common as asthma and 7 times as common as hay fever reported in children aged 5 to 17 years old (1). Surveillance for dental caries, dental sealants, tooth retention, edentulism, and enamel fluorosis by the National Health and Nutrition Examination Survey (NHANES) during 1988-1994 and 1999-2002 reported that despite decrease of caries prevalence in permanent dentition, the caries prevalence in primary dentition is increasing and still a concern (2). Moreover, oral health disparities among US population are another major concern contributing to the problem of dental caries. Children with low socioeconomic status had more untreated carious teeth than children with high socioeconomic status. Furthermore, there are also problems with access to care that contribute to disparities among ethnicity in the group of low socioeconomic status children (2). Consequently, caries-risk assessment has been proposed as an essential tool for targeting children at risk to provide specific treatment plan and caries preventive plan for population groups as well as individuals. This assessment involves consideration of individual factors that contribute to or protect from caries. Caries-risks factors for children include frequent
exposure to sugar-containing snacks or beverages, caries experience, mother’s caries status, special health care needs, socioeconomic status, immigration status, oral hygiene and elevated mutans streptococci levels. Caries protective factors include optimal systemic and topical fluoride exposure, proper oral hygiene and regular access to dental care (3, 4).

Although infectious, dental caries is multifactorial (i.e., dietobacteria) making it a complex chronic disease. Data suggest that mutans streptococci (MS) play an important role in the initiation and development of dental caries (5, 6). Human MS species are *Streptococcus mutans* and *Streptococcus sobrinus*. These bacterial species have been reported to be detected in oral samples, such as plaque, saliva and tongue scrapings (7, 8). *S. mutans* is found to be the most common streptococcal species isolated from human oral cavity (7, 8). It was mostly accounted for 74% to 100% of the human isolates of MS from various populations (7). Studies show that the level of *S. mutans* from clinical samples, such as dental plaque and saliva, is highly associated with dental caries prevalence (9-13). *S. sobrinus* is also a common streptococcal species isolated from human oral cavity. In this regard, it has been reported to be detected in 0% to 53% of the human isolates of MS of samples from various populations (7). The level of *S. sobrinus* in oral samples is also strongly associated with caries prevalence (10, 12). Not only MS counts that has been found to be associated with dental caries, ratios of MS to total cultivable bacteria has also been found to be an important factor of carious lesion initiation and progression. Studies have shown high correlation between the ratio of *S. mutans* to total bacteria in clinical samples and prevalence of dental caries as well as ratio of *S. sobrinus* to *S. mutans* and prevalence of dental caries (9, 10, 13-16).
Detection of MS in the oral cavity can be important information to help predict caries risk (9, 10, 14-17). It has been reported that the earlier the MS colonize children, the higher the caries prevalence (11, 18-20). Several methods have been used to detect MS from oral samples, for example; direct microscopy, cultivation, enzyme test, monoclonal antibodies (mAbs), enzyme-linked immunosorbent assays (ELISA), species specific DNA probes such as conventional end-point polymerase chain reaction (PCR) (9, 10, 14-16). Most of these methods are time-consuming, laborious, and relatively unspecific (10).

Bacterial culture on specific media is a routine method in detection and quantification of MS although it does not reveal diversity of bacterial biofilm communities. Traditionally, Mitis salivarius agar and Mitis salivarius-bacitracin agar are used for culturing MS. By this method, S. mutans and S. sobrinus can be distinguished from one another by differences of colonial morphology (9, 21, 22).

Polymerase chain reaction (PCR) is also used for detection and quantification of MS. The advantages of PCR are high sensitivity and specificity in detection of oral bacteria of interest (23, 24). In order to quantify bacterial species in biological samples, real-time quantitative PCR (qPCR) was introduced as a novel method for rapid detection and quantification of bacterial species (25). Using sequence-specific primers of a particular DNA sequence of genes of interest, bacterial numbers can be quantified from the amplification of double stranded DNA products. While performing PCR cycles, the amplified DNA products are bound by fluorescent DNA dyes, allowing determination of relative number of copies of DNA.
Several studies using qPCR for detection and quantification of MS from oral samples reported a high association between MS findings and caries prevalence (9-11, 13, 14, 26). The samples from these studies were collected from populations in Asia (9-11, 13), Africa (14) and Haiti (26). None of the studies were from North America. The purpose of this study was to use qPCR and standard plate counting (SPC) for detection and quantification of levels of *S. mutans*, *S. sobrinus* and total streptococci in clinical samples from children considered to be at high-caries-risk because there is no regular access to dental care in the county they reside. Furthermore, levels of MS and total streptococci were evaluated for correlation with caries prevalence.

**MATERIAL AND METHODS**

**Study Population**

Subjects in this study were part of an ongoing longitudinal epidemiological study from a high-caries-risk community in Perry County, Alabama. Fifty-eight school-aged children who live in this rural county in the Black Belt of Alabama with no regular access to dental care participated in this study. Because of the poor access to care, this population is considered high caries risk.

Inclusion criteria originally was children at age 8-10 years old who are free of systemic diseases, i.e., genetic and birth defects, kidney disorders, hepatitis, cancer, bleeding disorders, endocrinial disorders, epilepsy, immunodeficiencies, heart conditions or murmurs, bone disorders, HIV positive, immunosuppressive drugs users. The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review
Board. Parents of participating subjects were provided with and had signed a consent form, while children gave assent for sample collection, oral examination and questionnaire completion.

**Oral Examination**

Oral examination by trained and calibrated examiners was performed using light source, compressed air source, mirror and explorer (limited for confirmatory use only); no radiographs were taken. Dental status was diagnosed and scored as decay, missing, filled tooth/surface score (i.e., DMFT/dmft and DMFS/dmfs) according to WHO criteria (27).

**Sample Collection**

Two types of clinical samples: plaque and saliva samples were collected from children during the same time of the day (i.e., mid-morning).

Plaque samples were collected using a sterile toothpick, scraping plaque from the occlusal, mesial, distal, buccal and lingual surfaces of prior selected tooth (mainly first permanent molar in school-aged children). Plaque was placed into 1 ml sterile reduced transport fluid (RTF) for processing.

Saliva samples were collected by providing paraffin for children to chew and spit saliva (approximately 5 ml) into a 50 ml sterile tube. Five hundred microliters of whole saliva sample was diluted 1:10 with 4.5 ml of RTF prior to processing.
After collection, all samples were stored on ice while being transported approximately 100 miles to UAB School of Dentistry. All samples were processed within 24-36 hours of collection.

**Isolation of DNA from Oral Samples**

One milliliter of oral sample was used for DNA extraction and purification with MasterPure™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer’s instruction as previously report by Childers, et al (28). After isolation, the DNA was re-suspended in 35 µl of Tris-EDTA buffer (TE buffer).

**PCR Primers**

The extracted DNA was then used as a template for qPCR using specific primers for *S. mutans*, *S. sobrinus* and total streptococci. The specificity of these primer sequences for *S. mutans* and *S. sobrinus* were confirmed by Yoshida *et al.* (29). The target gene for *S. mutans* primer was gtfB and for *S. sobrinus* was gtfT. The streptococcal elongation factor Tu gene, tuf, was used to design “universal” primers that are specific for and unique to streptococcal species using Plexor analysis software from Promega (Promega Corporation, Madison, WI, USA). This process yielded a set of streptococcal species- specific primers that generate 99bp amplicons that of the appropriate size for qPCR analysis. The sequence of the forward gtfB primer for *S. mutans* detection, Smut3368-F, was 5’-GCCTACACGCTAGAGATGCTATTCT-3’, and the sequence of the reverse gtfB primer, Smut3481-R, was 5’-GCCATACACCACTCATGAATTGA-3’. Amplification of *S. mutans* genomic DNA with primers Smut3368-F and Smut3481-R
produces a 114-bp amplicon. The sequence of the forward gtfT primer for *S. sobrinus* detection, Ssob287-F, was 5’-TTCAAGCCAAGCAAGCTAGT-3’, and the sequence of the reverse gtfT primer, Ssob374-R, was 5’-CCAGCCTGAGATTCAGCTGTTTCTGT-3’. Amplification of *S. sobrinus* genomic DNA with primers Ssob287-F and Ssob374-R produces a 88-bp amplicon. The sequence of the forward primer for TS detection, Plexor Quadraplex T. Strep F was 5’-GGATTTCATTTCAACCAATTCAAGCAA-3’, and the sequence of the reverse primer, Plexor Quadraplex T. Strep R was 5’-ACATCCTTCTTTCACGSTGTTG-3’. The specificity of all the primer sequences for mutans streptococci and total streptococci was confirmed through Basic Local Alignment Search Tool (BLAST) nucleotide analysis, focusing on known human commensal oral bacteria that would not serve as a template.

**Real-time Quantitative PCR (qPCR)**

Real-time quantitative polymerase chain reactions (qPCR) were performed as previously reported (Childers *et al.*, 2011) (28) using a matrix of forward primer, reverse primer, and fluorescent dye to determine the optimal concentration yielding the lowest quantification cycle (Cq) values and, hence, the highest amplification efficiencies.

*S. mutans* UA 159 genomic DNA at 10⁹ cells ml⁻¹, which was determined by a Bio-Rad Smart Spec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA), was ten-fold diluted with TE buffer from MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) to concentration at 10³ cells ml⁻¹. These known concentrations of *S. mutans* UA 159 genomic DNA were used to construct a
standard curve for each qPCR run for estimation of the “copy number” per milliliter (CN/ml) of *S. mutans*, *S. sobrinus* and total streptococci from Cq values. *S. mutans* UA 159 and *S. sobrinus* 6715 were used as positive and negative control.

PCR amplification was performed in a total reaction mixture volume of 12.5 µl. The reaction mixtures contained 6.25 µl Maxima® SYBR Green/Fluorescein qPCR Master Mix (Fermentas, USA), 0.75 µl of forward and reverse primer, 3 µl of nuclease-free water and 2.5 µl of purified DNA obtained from oral sample. The reagents and reaction conditions used in the standard curve were the same as those used for oral samples mixtures for enumeration of *S. mutans*. For enumeration of *S. sobrinus* and total streptococci, the qPCR reaction mixture of the oral sample DNA used were the same as for the qPCR for *S. mutans* with UA 159 genomic DNA in mixture for standard curves.

An iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used to perform the following cycling parameters: 1 cycle of denaturing at 95°C for 10 minutes, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds of 72°C. A final melting curve program used the following cycling parameters: 60°C for 30 seconds, 5°C temperature changes to the end temperature of 95°C. All output data were analyzed using iQ5 Optical System Software version 2.1 (Bio-Rad). A linear standard curve was generated from a plot of log concentration of the known concentration of *S. mutans* UA 159 genomic DNA against quantitative cycle (Cq) with high correlation coefficient (*R*² = 0.99 – 1.00) as shown in Figure 1. Melting curves for targeted bacterial species demonstrated a sharp peak at the expected melting temperature (Tm), indicating that qPCR products were homogeneous without primer-dimers and the chosen primers were specifically amplified the targeted DNA. Tm for *S. mutans*, *S. sobrinus* and total
streptococci were at 74°C, 81°C and 76°C, respectively as shown in Figure 2a and 2b. The Cq from qPCR of the oral samples were extrapolated on the generated standard curve for estimation of the “copy number” per milliliter (CN/ml) of S. mutans, S. sobrinus and total streptococci. All qPCRs for the oral samples and the standards were performed in duplicate. The average of the two reaction results were used in final statistical analysis.

**Standard Plate Counting**

Saliva samples in RTF were vortexed whereas plaque samples were sonicated for 20 s (Vibra cell™, Sonics & Materials Inc., Danbury, Conn., USA) before being plated on selective media for determination of the number of colony forming units per milliliter (CFU/ml). Fifty microliters of several 10-fold dilutions for each sample was plated using spiral plater (Spiral Systems Inc., Cincinnati, Ohio, USA) on duplicate plates of mitis salivarius agar (Difco™ Mitis Salivarius Agar, Becton, Dickinson & Co., Sparks, Md., USA) for enumeration of total streptococci and Gold’s agar plates (30), which consists of mitis salivarius agar plates supplemented with bacitracin for enumeration of mutans streptococci. Following anaerobic incubation (10% carbon dioxide, 10% hydrogen, and 80% nitrogen) at 37°C for 48 hours, S. mutans (Sm), S. sobrinus (Ss) and total streptococci (TS) were counted according the spiral plater instruction to calculate CFU/ml from each duplicated plate. The average counts of the two plates, each were used in final statistical analysis.

**Statistics**

The mean levels of S. mutans (Sm), S. sobrinus (Ss) and total streptococci (TS) from duplicate clinical samples obtained from qPCR and SPC were used for quantitative
analysis. Additionally, the ratio of CN/ml and CFU/ml of *S. mutans* to total streptococci (*Sm/TS*) from each sample were calculated. The mean of these ratios were used for the final analysis. Pearson’s correlation analysis and t-test were used to evaluate the correlation between mean bacterial level, mean bacterial ratio and caries prevalence (DMFS/dmfs), and caries status of subjects (had caries experience or caries-free) using SAS V9.2 (SAS Institute, Cary, NC, USA).

**RESULTS**

Fifty-eight school-aged children (33 males, 25 females) with average age of 9.76 years (age range 8.87 - 10.67 years) participated in the study. All subjects were African American. Fifty-eight saliva samples were collected from each subject to use for qPCR and SPC. Ninety-nine plaque samples were collected for qPCR analysis whereas 75 plaque samples were collected for SPC analysis. The detection threshold of this qPCR was determined to be 70 CN/ml whereas of SPC was 400 CFU/ml (28).

*Sm* was detected by qPCR from 98.1% of samples (154/157) while *Ss* was only found in 2.6% of samples (4/157). By SPC, *Sm* was detected from 72.9% (97/133) of samples while none of *Ss* was detected (Table 1). Mean qPCR CN/ml of samples were: *Sm* (2.54x10^6), *Ss* (5.27x10^2) and TS (2.34x10^9). Mean SPC CFU/ml of samples were: *Sm* (0.94x10^6), *Ss* (0) and TS (0.01x10^9).

The average DMFS/dmfs score of the participants was 6.97 (DMFS/dmfs range 0-40). Among them, 28 subjects (48%) were caries free (DMFS/dmfs=0). For saliva samples, significant correlations by Pearson’s correlation analysis were found between
the level of \( Sm \) from PCR to DMFS/dmfs \((r=0.47, P=0.0002)\), level of \( Sm \) from SPC to DMFS/dmfs \((r=0.30, P=0.0216)\), ratio of \( Sm/TS \) from PCR to DMFS/dmfs \((r=0.34, P=0.01)\) and ratio of \( Sm/TS \) from SPC to DMFS/dmfs \((r=0.36, P=0.0057)\) (Table 2).

For plaque samples, significant correlations by Pearson’s correlation analysis were found between level of \( Sm \) from PCR to DMFS/dmfs \((r=0.26, P=0.0085)\). However, there was no significant correlation between level of \( Sm \) from SPC to DMFS/dmfs \((r=0.052, P=0.6571)\). Furthermore, significant correlations were found between ratio of \( Sm/TS \) from PCR to DMFS/dmfs \((r=0.41, P=<.0001)\), ratio of \( Sm/TS \) from SPC to DMFS/dmfs \((r=0.36, P=0.0016)\) (Table 2).

Interestingly, significant correlations were found between ratio of \( Sm/TS \) and active caries in both primary dentition and permanent dentition. This was shown by significant Pearson’s correlation value observed between decayed teeth to DMFS/dmfs as well as between decayed surfaces to DMFS/dmfs in both primary and permanent dentition (Table 3).

When comparing two groups of children according to caries status, children who had caries experience \((DMFS/dmfs > 0)\) had a higher level of \( Sm \) and higher ratio of \( Sm/TS \) than children who were caries-free \((DMFS/dmfs = 0)\). These higher levels and ratios were observed in both types of samples and from both technique; qPCR and SPC. However, statistically differences between groups were found only in mean \( Sm \) and mean \( Sm/TS \) from saliva samples with qPCR (Table 4).
DISCUSSION

This study found that qPCR for detection of *Sm* from saliva samples was 100%, and was 97.9% of plaque samples (Table 1). SPC however was found to have lower sensitivity and detection threshold, (91.4% in saliva and 50.7% in plaque samples). Interestingly, *Sm* was detected almost twice as much in plaque samples by qPCR compared to SPC. Biologically, *Sm* is colonized and co-aggregated in dental biofilm in the oral cavity. This co-aggregated consortium of *Sm* (*including* other plaque bacteria) can impact the accuracy of SPC *Sm* detection due to inadequate cell dispersion for cell separation to result in individual CFU growth. Effort to control this limitation is to sonicate or to vortex oral samples prior to culture. However, this dispersion method can result in loss of cell viability. Therefore, loss of cell viability would likely explain the lower number of *Sm* growing on the selective media is another issue that may diminish colony growth. Also, loss of cell viability during transportation may another reason for lower detection rate of SPC compared to qPCR. The method of qPCR, however, does not rely on cell viability but rather DNA, which remains intact in the cell prior to extraction by cell lysis.

Prior to the initiation of qPCR for MS quantitation, very few samples were found to grow *Ss*. This finding was a source of concern that the selective media employed or the method of *Ss* colony identification was causing a lack of detection of *Ss*. In fact, most children in this study did not have detectable *Ss* by qPCR and SPC. As for *Sm*, qPCR has higher sensitivity than SPC, therefore, the minute detection rate of *Ss* by qPCR in both types of samples confirmed that the undetectable *Ss* by SPC resulted from lower level or absence of *Ss* in oral cavity of participants in this study. It is likely that the low sensitivity
of detection with SPC resulted from the rare occurrence of Ss in this cohort, which, if present or non-viable may explain why Ss was not identified on select media used in this study. Previous studies from Asia (9-11, 13), Africa (14) and Haiti (26) showed that Ss presence in oral samples were correlated to dental caries. As review by Loesche in 1986, Ss were detected for 0% to 53% from various populations. From this review, Ss were detected in only 0.4% of the United States population (7). Considering that the participants in this study were only African American, the difference of Ss detection among the present study and previous studies might be due to ethnicity of the study subjects. However, since some studies reported Ss in African populations (including Haiti), the lower Ss may be due to geographical location of this study. Interestingly, among 4 subjects who had detectable Ss in this study, only 1 of them had caries experience with DMFS/dmfs score of 13 (1 missing tooth, 1 decayed and 7 filled surfaces). This finding does not support previous studies that show strong correlation with the presence of Ss to dental caries.

When using qPCR and SPC for quantification of MS and TS (Table 2), the mean level of Sm, Ss and TS CN/ml by qPCR were higher than CFU/ml by SPC. As discussed previously, higher sensitivity of qPCR, clumping of MS cells and non-viability of the cells at the time of plating can be the reason why SPC generate lower CFU/ml than CN/ml from qPCR.

Because of low detection of Ss in the samples of this study, only mean levels of Sm and mean ratios of Sm/TS were analysed for correlation to caries data, i.e. caries prevalence (DMFS/dmfs) and active caries lesions (DT, DS, dt, ds). The mean levels of Sm in plaque samples were significantly correlated to caries prevalence (DMFS/dmfs).
when quantitated by qPCR, however, the correlation was not significant by SPC (Table 2). Additionally, neither of the mean levels of Sm in plaque samples by qPCR nor by SPC was associated with presence of active caries lesions (DT, DS, dt, ds) (Table 3). Because of the difficulty in controlling the quantity of plaque samples collected (31), and large variations of bacterial counts between plaque collected from different tooth surfaces (14), and different sampling methods (pooled or individual sample) (32), the ratio of Sm/TS was used in this study to normalize the data to evaluate correlation of Sm to dental caries. Similar to a previous study (9) that showed that the ratio of Sm/TS from decayed or filled, and decayed incisors were higher than from sound, and sound and/or filled incisors. Therefore, the results from the present study also showed that irrespective of sample types or techniques (i.e., qPCR or SPC) used for quantification of Sm and TS, the mean ratios of Sm/TS were significantly correlated to caries prevalence and active caries. It is important to note that plaque samples collected in this study were generally not from carious teeth, even if active caries was present. Consequently, for general screening, the ratio of Sm/TS maybe a more appropriate parameter than level of Sm when using plaque samples to evaluate MS and dental caries in future prospective studies of caries risk.

The findings of this study supported that the use of saliva samples as a surrogate for the oral biofilm content that is arguably the more relevant sample but difficult to consistently and reproducibly collect. In this study, saliva samples showed more consistent significant correlations between mean levels of Sm and mean ratios of Sm/Ts to caries prevalence and active caries lesions than plaque samples (Tables 2 and 3). This type of sample also gave higher detection rate of Sm and Ss irrespective of technique used for MS detection (i.e, both by qPCR and SPC, Table 1). Moreover, saliva samples were
the only samples that showed significant difference of mean levels of Sm and mean ratios of Sm/TS between caries-active and caries-free children (Table 4). Consequently, the use of saliva samples appear to be more appropriate to assess the presence of bacterial component (biofilm) in the oral cavity because bacteria in the saliva is a reflection of the biofilm on the teeth, and furthermore, may be a useful representation of all teeth in the dentition, when individual tooth sampling is not practical. As reported by others, therefore, our data indicate that salivary bacterial counts can be considered as a reflection of bacterial load from dental biofilm of individual’s oral cavity (14, 33).

In conclusion, detection and quantification of MS can be a useful method to predict caries risk. This study showed that the levels of Sm and ratio of Sm/TS by qPCR and SPC were correlated to existing dental caries. Although the data presented here found a correlation between quantitation of MS and existing history of dental caries, as well as active caries, the detection by the qPCR were comparable to SPC. SPC technique is more susceptible to sampling methods, i.e. processing, storage and growth on selective media, as well as more time and labor intensive. Given the lower limit of detection and similar correlation found, the more streamlined method of qPCR is a suitable outcome measure in epidemiological studies.

ACKNOWLEDGEMENTS

The authors wish to thank all the clinical and laboratory participants of this study: Ms Stephanie McLean, Dr Steve Mitchell, Dr Sonia Makhija, Dr Rosalyn Bassett, Ms Mary Slater, Ms Frances Jackson, and the pediatric dental residents of the UAB School of
Dentistry. This study was supported by Department of Pediatric Dentistry, University of Alabama at Birmingham, School of Dentistry and a Research Grant DE016684 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA. Dr. Manmontri received a Dr. Britta Rahemtulla Endowed Scholarship Fund Award for Achievement in Research and a scholarship from the UAB Graduate School Fellowship Program, School of Dentistry, University of Alabama at Birmingham.

**LIST OF REFERENCES**


(18) Kohler B, Andreen I, Jonsson B. The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age. Oral Microbiol Immunol 1988: 3: 14-17.


**Table 1:** Detection of *Sm* and *Ss* from oral samples by qPCR and SPC.

<table>
<thead>
<tr>
<th></th>
<th>Sm</th>
<th>Ss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>qPCR</td>
<td>SPC</td>
</tr>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td><strong>Saliva samples</strong></td>
<td>58/58 (100%)</td>
<td>0/58 (0%)</td>
</tr>
<tr>
<td><strong>Plaque samples</strong></td>
<td>96/99 (97.0%)</td>
<td>3/99 (3.0%)</td>
</tr>
<tr>
<td><strong>All samples</strong></td>
<td>154/157 (98.1%)</td>
<td>3/157 (1.9%)</td>
</tr>
</tbody>
</table>

*Sm, Streptococcus mutans; Ss, Streptococcus sobrinus; qPCR, real-time quantitative PCR; SPC, standard plate counting.*
Table 2: Quantification of Sm and ratios of Sm/TS from oral samples by qPCR and SPC and correlation to caries index.

<table>
<thead>
<tr>
<th></th>
<th>qPCR</th>
<th></th>
<th></th>
<th>SPC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saliva samples (n=58)</td>
<td>Plaque samples (n=99)</td>
<td></td>
<td>Saliva samples (n=58)</td>
<td>Plaque samples (n=75)</td>
</tr>
<tr>
<td>Mean Sm</td>
<td>5.42x10^6</td>
<td>0.47 (0.0002**)</td>
<td>8.56x10^5</td>
<td>0.26 (0.0085*)</td>
<td>1.93x10^6</td>
<td>0.30 (0.0216*)</td>
</tr>
<tr>
<td>Mean Sm/TS</td>
<td>3.71x10^-3</td>
<td>0.34 (0.01*)</td>
<td>4.09x10^-2</td>
<td>0.41 (&lt;.0001**)</td>
<td>2.64x10^-1</td>
<td>0.36 (0.0057*)</td>
</tr>
</tbody>
</table>

Sm, Streptococcus mutans; TS, total streptococci; qPCR, real-time quantitative PCR; SPC, standard plate counting; CFU, colony-forming units; CN, copy number.

*p<0.05, significant correlation, Pearson’s correlation analysis

**p<0.001, significant correlation, Pearson’s correlation analysis
Table 3: Quantification of Sm and ratios of Sm/TS from oral samples by qPCR and SPC and correlation to active caries.

<table>
<thead>
<tr>
<th></th>
<th>Saliva samples</th>
<th>Plaque samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permanent dentition</td>
<td>Primary dentition</td>
</tr>
<tr>
<td></td>
<td>DT</td>
<td>DS</td>
</tr>
<tr>
<td>Mean Sm from qPCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>(&lt;.0001**)</td>
<td>(&lt;.0001**)</td>
</tr>
<tr>
<td>Mean Sm from SPC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.39519</td>
<td>0.13212</td>
</tr>
<tr>
<td></td>
<td>(0.0021*)</td>
<td>(0.3228)</td>
</tr>
<tr>
<td>Mean Sm/TS from qPCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>(0.0006**)</td>
<td>(0.0006**)</td>
</tr>
<tr>
<td>Mean Sm/TS from SPC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>(0.0117*)</td>
<td>(0.0112*)</td>
</tr>
</tbody>
</table>

Sm, Streptococcus mutans; TS, total streptococci; qPCR, real-time quantitative PCR; SPC, standard plate counting.

Row and column definition: DT=decayed teeth in permanent dentition, DS=decayed surfaces in permanent dentition, dt=decayed teeth in primary dentition, ds=decayed surfaces in primary dentition

*p<0.05, significant correlation, Pearson’s correlation analysis

** p<0.001, significant correlation, Pearson’s correlation analysis
Table 4: Quantification of Sm and ratios of Sm/TS from oral samples by qPCR and SPC and T-test analysis to caries status.

<table>
<thead>
<tr>
<th>qPCR</th>
<th>Saliva samples</th>
<th>Plaque samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMFS/dmfs &gt; 0 (n=30)</td>
<td>DMFS/dmfs = 0 (n=28)</td>
</tr>
<tr>
<td>Mean Sm (CN/ml)</td>
<td>9.23 x 10^6</td>
<td>1.35 x 10^6</td>
</tr>
<tr>
<td>Mean Sm/TS</td>
<td>6.04 x 10^-3</td>
<td>1.22 x 10^-3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SPC</th>
<th>Saliva samples</th>
<th>Plaque samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMFS/dmfs &gt; 0 (n=30)</td>
<td>DMFS/dmfs = 0 (n=28)</td>
</tr>
<tr>
<td>Mean Sm (CN/ml)</td>
<td>3.39 x 10^6</td>
<td>3.62 x 10^5</td>
</tr>
<tr>
<td>Mean Sm/TS</td>
<td>4.92 x 10^-1</td>
<td>1.97 x 10^-2</td>
</tr>
</tbody>
</table>

Sm, Streptococcus mutans; TS, total streptococci.

Row and column definition: (DMFS/dmfs > 0)=children who have had caries, (DMFS/dmfs = 0)=children who were caries-free

*p<0.05, significant correlation, T-test analysis
FIGURE LEGENDS

Figure 1 legend: Standard curve of a ten-fold dilution series of known template concentrations of *S. mutans* UA 159. Standard curve of a ten-fold dilution series of known template concentrations of *S. mutans* UA 159 (10⁹ CN/ml to 10³ CN/ml) for determining the initial starting amount of the *S. mutans* in each oral sample. The log of each known concentration in the dilution series (x-axis) is plotted against the threshold cycle or quantitative cycle value (Cq) for that concentration (y-axis). Correlation coefficient (R²) of 0.99 – 1.00 from this standard curve reflects the linearity of the curve.

Figure 2a legend: Melting curve chart for *S. mutans* and total streptococci. Melting curve chart for the targeted oral bacteria demonstrated a sharp peak at the expected melting temperature (Tm); *S. mutans* (74°C) and total streptococci (76°C), indicating that qPCR products were homogeneous without primer-dimers artifacts and contamination. Also, the chart ensures the specificity of the chosen primers in this primer which specifically amplified the targeted DNA.

Figure 2b legend: Melting curve chart for *S. mutans* and *S. sobrinus*. Melting curve chart for the targeted oral bacteria demonstrated a sharp peak at the expected melting temperature (Tm); *S. mutans* (74°C) and *S. sobrinus* (81°C), indicating that qPCR products were homogeneous without primer-dimers artifacts and contamination. Also, the chart ensures the specificity of the chosen primers in this primer which specifically amplified the targeted DNA.
Figure 1: Standard curve of a ten-fold dilution series of known template concentrations of *S. mutans* UA 159
Figure 2a: Melting curve chart for *S. mutans* and total streptococci
Figure 2b: Melting curve chart for *S. mutans* and *S. sobrinus*
GENERAL CONCLUSIONS

Dental caries is an infectious disease that affects most populations around the world, especially children. Dental caries can adversely impact children’s weigh, growth, and well-being. Although preventable, caries is the most prevalent infectious childhood disease in United States. Caries is multifactorial (i.e., dietobacteria) making it to be considered as complex chronic disease. Factors associated with caries are including host factors (tooth surface, saliva, and acquired pellicle), diet, and dental plaque (bacteria). Data suggest that mutans streptococci (MS), i.e. *Streptococcus mutans* and *Streptococcus sobrinus*, play an important role in the initiation and development of dental caries (3, 4). Many studies reported that MS are dominantly detected in oral samples, such as plaque, saliva and tongue scraping samples (5, 6) and MS level and proportion in total cultivable bacteria have strong correlation to dental caries(3, 7). Traditional bacterial cultures on selective media were mostly used in these previous studies for quantitatively and qualitatively assessing MS colonization in oral cavity. However, the main disadvantages of this method are: time-consuming, labor-intensive, and impractical in field epidemiological studies (9, 19, 27, 29). Moreover, SPC is susceptible to biological limitation of oral samples, for example; loss of cell viability during samples processing or inadequate cell dispersion prior to sample plating can result in inaccurate detection and quantification of MS (36, 38). Consequently, these limitations prevent traditional culture
methods from providing accurate assessment of the microbial load during evaluation of caries susceptibility or caries activity in individuals or in a population at risk.

Real-time quantitative PCR (qPCR) was introduced as a novel method to quantify bacterial species in biological samples providing rapid detection and quantification (32). The major advantage of qPCR is that it overcomes inability of detecting non-viable bacteria in culture method eliminating some of the critical concern of conventional bacterial culture about clinical sample handling, transportation and storage (11, 29, 33). Also, based on primer design, qPCR is highly specific and sensitive. This technique has been shown to be more sensitive than conventional bacterial culture in detection of targeted bacterial species (8, 9, 30, 34-36). These advantages of qPCR assay make it to be an excellent tool and a practical method for an epidemiologic study with a large-scale population (11, 29).

The results from studies in this thesis show that qPCR usually detected a higher quantity of Sm CN/ml than Sm CFU/ml from SPC. Furthermore, with the methods of this study, qPCR has a lower detection threshold than SPC (70 CN/ml compared to 400 CFU/ml, respectively). The qPCR technique overcomes biological limitation of SPC which failed to detect and quantify Sm cells compared to qPCR. Many potential advantages can be proposed to explain the findings of this study. qPCR does not appear to be limited by inadequate cell separation or loss of cell viability that may occur prior to sample processing. Moreover, qPCR showed more significant correlations between the levels of Sm and ratio of Sm/TS to dental caries, i.e. caries prevalence (DMFS/dmfs) and active caries lesions (DT, DS, dt, ds). These correlations when using qPCR to quantify MS and TS was higher and provide better consistency of significant correlations when
compared to using SPC for MS quantification. Interestingly, the presence of Ss in this high caries risk population is very rare and there is no association with caries prevalence.

It is not only Sm colonization that is important in clinical caries risk assessment: accurate sample is also another important factor that clinicians should consider. Saliva samples and plaque samples are the most common sampling method used for a clinical caries assessment. From studies reported in this manuscript, saliva collected from children expectorating into a cup generally resulted in higher detection rate of MS, higher counts of Sm, and less discrepancy between detection of Sm than other types of samples, irrespective of the technique used for MS detection (qPCR or SPC). Moreover, saliva sample showed more consistent significant correlations between mean levels of Sm and mean ratios of Sm/Ts to caries prevalence and active caries lesions when comparing to plaque samples. Additionally, saliva samples were more useful than plaque in demonstrating significant differences in mean levels of Sm and mean ratios of Sm/TS by qPCR between caries-active and caries-free children. Therefore, the use of saliva samples appear to be the most qualitatively and quantitatively consistent and would be the best type of sample for future study related to MS colonization in oral cavity.

In conclusion, the findings from this Master’s degree project indicate that qPCR has a potential to replace SPC in detection and quantification of MS, which would ultimately be a useful method to predict caries risk. Given the lower limit of detection, higher sensitivity, high specificity, less susceptibility to sampling methods, less labor and laboratory intensive coupled with the finding of similar correlations to dental caries, the more streamlined method of qPCR is a suitable outcome measure for design of future longitudinal epidemiological studies. Future goals will be focused on evaluation of
qualitative and quantitative data of MS and possible other caries-associated bacterial species, and their association with dental caries and caries risk prediction.
GENERAL LIST OF REFERENCES


(10) Loyola-Rodriguez JP, Martinez-Martinez RE, Flores-Ferreyra BI, Patino-Marin N, Alpuche-Solis AG, Reyes-Macias JF. Distribution of Streptococcus mutans and Streptococcus sobrinus in saliva of Mexican preschool caries-free and caries-


(22) Kohler B, Andreen I, Jonsson B. The earlier the colonization by mutans streptococci, the higher the caries prevalence at 4 years of age. Oral Microbiol Immunol 1988: 3: 14-17.


APPENDIX

IRB APPROVAL FORMS
Project Revision/Amendment Form

Today's Date: April 26, 2010

1. Contact Information
   Principal Investigator's Name: Noel Childers, DDS  BlazerID: nkc E-mail: nkc@uab.edu
   Contact Person's Name: Stephanie McLean  BlazerID: smclean E-mail: smclean@uab.edu
   Telephone: 996-6628  Fax: 934-7013
   Campus Address: SDB 304B

2. Protocol Identification
   Protocol Title: Epidemiology of Dental Caries and Immunity in Children (Alabama)
   IRB Protocol Number: F060328001
   Current Status of Project (check only one):
   ☑ Currently in Progress (Number of participants entered: 868)
   □ Study has not yet begun (No participants entered)
   □ Closed to participant enrollment (remains active)—
     Number of participants on therapy/intervention:
     Number of participants in long-term follow-up only:
     Total number of participants enrolled:
   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
   □ Revised Consent Form
   □ Addendum (new) consent form
   □ Study Closure
   □ Enrollment temporarily suspended by sponsor
   □ Change in protocol personnel
   □ Other, (specify) ____________________________

3. Reason for change
   We are adding the 1st Year Pediatric Dentistry Residents to our list of study personnel: Lenora Covington, Elizabeth Evans,
   Chaneke Manifest, Mahendra Mavalli, James Osborn and Kyle Reed. Their completed IRB Human Subjects Protection training
   verification is attached.

4. Does this change revise or add a genetic or storage of samples component?
   ☑ Yes  ☐ No

5. Does the change affect subject participation (e.g., procedures, risks, costs, location of services, etc.)?
   ☑ Yes  ☐ No
   If yes, Fiscal Approval Process (FAP)-designated units complete a FAP submission and send to
   fap@uab.edu. For more on the UAB FAP, see www.uab.edu/ohr.

6. Does the change affect the consent document(s)?
   ☑ Yes  ☐ No
   If yes, briefly discuss the changes.
   Include the revised consent document with the changes highlighted.
   Will any participants need to be reconsented as a result of the changes?
   □ Yes  ☐ No

Signature of Principal Investigator: Noel Childers Date: 4/26/10

224 - Amendment 19.doc
10/15/08

APPROVED
KESSEL, CIP
IRB REVIEWER
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 (56 FR 30003, 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 appropriate Institutional Review Board (IRB)
review and approval to the Department or Agency in accordance with the Common Rule.

1. Request Type
   [ ] ORIGINAL
   [ ] CONTINUATION
   [X] EXEMPTION
   [ ] OTHER:

2. Type of Mechanism
   [ ] GRANT
   [ ] CONTRACT
   [ ] FELLOWSHIP
   [ ] COOPERATIVE AGREEMENT
   [ ] OTHER:

3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.

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)
   [ ] This Assurance, on file with Department of Health and Human Services, covers this activity:
     Assurance No. PWA0000000000, the expiration date 12/20/2019, IRB Registration No. IRB000000726
   [ ] This Assurance, on file with (agency/site), the expiration date 09/01/2020, Assurance No. PWA0000000000,
     covers this activity.
   [ ] 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)
   [ ] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
   [ ] If less than one year approval, provide expiration date 11/11/2010
   [ ] 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
   Protocol subject to Annual continuing review.

   Title P506328001
   Epidemiology of Dental Caries and Immunity in Children (Alabama)

9. IRB Approval issued:
   12/08/09

10. Name and Address of Institution
    University of Alabama at Birmingham
    701 20th Street South
    Birmingham, AL 35204

11. Phone No. (with area code) (205) 934-3789
12. Fax No. (with area code) (205) 934-1301
13. Email: smoore@uab.edu

14. Name of Official
    Ferdinand Unthaler, M.D.

16. Signature
    Ferdinand Unthaler, M.D.

17. Date 12/08/09

Public reporting burden for this collection of information is estimated to average less than an hour per response. An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to OMB Control Office, Room 3503/3700, Washington, D.C. 20503. Do not return the completed form to this address.