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Quantification of Salivary *Streptococcus mutans* by *q*PCR

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Quantification of Salivary *Streptococcus mutans* by *q*PCR

Amy Chen

Abstract

Purpose: To develop a SYBR® Green *q*PCR method for quantifying *Streptococcus mutans* (SM) in salivary samples.

Methods: SM specific primers were derived from the DNA sequence of AP-PCR amplification products (unpublished). Serial dilutions of overnight mutans streptococci (MS) cultures were blotted on FTA cards (Whatman Bioscience Ltd.) as standards for *q*PCR. Sixty clinical saliva and swab samples, from children aged 2-10 with or without caries, enumerated for SM by traditional culturing were archived on FTA cards for SM quantification by SYBR® Green *q*PCR. The specificity of *q*PCR was analyzed by product melting curves. Correlation of SM quantification by *q*PCR and culture was analyzed using Pearson correlation test. The correlation of MS quantification by culture, or SM quantification by *q*PCR, and caries scores were analyzed by Pearson correlation test.

Results: A total of 60 clinical samples (20 saliva and 40 swab samples) were enumerated by traditional culturing, and analyzed by *q*PCR. The 20 saliva samples were from caries-active (high caries risk) children, and the 40 swab samples were from both caries-active and caries-free children. The SYBR® Green *q*PCR method provided high efficiency and good specificity for SM detection in salivary samples archived on FTA cards. *q*PCR showed good reproducibility with a mean variance (CV) = 0.13 for *q*PCR samples with $\log_{10}$ SM > 3 CFU/ml. SM quantification by *q*PCR was significantly correlated (Pearson's correlation) with MS culture enumeration for all clinical samples, ($r=0.51$, $P<0.001$), saliva samples ($r=0.52$, $P<0.05$), and swab samples ($r=0.51$, $P<0.001$).

In addition for swab samples, *q*PCR and culture results were significantly correlated with caries status (DS, $r=0.32$, $P<0.05$ for *q*PCR; DMFS and DS $r=0.51$, $P<0.001$ and $r=0.44$, $P<0.01$, respectively for culture).

Conclusions: *q*PCR quantification of SM showed good correlation with traditional culture results. It offers a fast and convenient quantification method for SM in clinical samples and could facilitate SM quantification for private practitioners. Development of *q*PCR techniques to quantify other cariogenic bacteria is needed for thorough evaluation of bacterial challenge.

TABLE OF CONTENTS

List of Figures

1. INTRODUCTION

Dental caries is the single most common chronic childhood disease, occurring five to eight times as frequently as the second-ranked asthma.[1] In 2000, the Unites States Surgeon General reported that 80% of caries occur in 25% of US children, concentrated in low income minority populations.[2] In 2005, the California Oral Health Needs Assessment reported that 25% of elementary school children have untreated decay.[3] The Center for Disease Control (CDC) in 2007 stated that tooth decay in 2-5-year-olds increased from 24% in 1988-94 to 28% in 1999-2004.[4] Despite the increased use of fluorides and other prevention regimens, the caries incidence appears to be increasing especially in families of low socioeconomic status with limited resources and access to care. This public health dilemma illustrates the fundamental need to better understand, develop, and implement caries prevention methods.

2. BACKGROUND

2.1 Dental Caries

Caries is a bacterial disease resulting from interaction among acidogenic bacteria, fermentable carbohydrates metabolized by microorganisms to acids, and tooth surfaces that are susceptible to acid dissolution. While the caries process involves a combination of factors, including diet and a susceptible host that interplay in a variety of social, cultural and behavioral factors, the type and quantity of microflora undeniably play a significant role. Mutans streptococci (MS), most commonly species *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) in humans, have been implicated in the etiology and progression of dental caries.[5, 6] *S. mutans* and *S. sobrinus* are gram-positive facultative bacteria that are both acidogenic and aciduric. They produce extracellular polysaccharides, which facilitate the

adhesion of microorganisms and the formation of plaque or biofilm on tooth surface. *S. mutans* and *S. sobrinus* metabolize many types of sugars to produce acid, mainly lactic acid that demineralizes tooth structure leading to tooth decay.[5, 7]

Early childhood caries (ECC) is the presence of 1 or more decayed (non-cavitated or cavitated lesions), missing (due to caries) or filled smooth surfaces in any primary tooth in a child 71 months of age or younger.[8] In children younger than 3 years of age, any sign of smooth-surface caries is indicative of severe early childhood caries (S-ECC).[8] From ages 3 to 5, 1 or more cavitated, missing (due to caries), or filled smooth surfaces in primary maxillary anterior teeth, or a decayed, missing or filled score of >4 (age 3), >5 (age 4), or >6 (age 5) surfaces constitutes S-ECC.[8] ECC can develop aggressively soon after dental eruption, progress rapidly and have a lasting harmful impact on the dentition. There is substantial evidence that children with caries as infants or toddlers have an increased chance of subsequent caries in the primary or permanent teeth.[9-11]

2.2 Caries Risk Assessment

Caries risk assessment involves the determination of the balance of pathological factors (i.e. frequent daily consumption of fermentable carbohydrates, reduced salivary function, high levels of salivary mutans streptococci) and protective factors (i.e. topical fluoride, anti-bacterial agents, adequate salivary flow) in the caries process.[7] Caries management by risk assessment is integral in the overall clinical decision making process and treatment plan. Levels of MS have been shown to be positively correlated with caries risk.[12, 13] A cross-sectional study involving 146 children indicated that those with $\log_{10}$ MS levels ≥ 3 were 5 times more likely to have ECC than those with lower bacterial levels.[14] In a longitudinal study of 39 children from 2 to 4 years of age, investigating the colonization of *S. mutans* in saliva and plaque with reference to caries

experience, it was determined that early establishment of *S. mutans* indicates a high risk of caries in young primary dentition.[13] In that same study, 5 out of 39 children who harbored *S. mutans* by the age of 2 experienced 60% of the lesions of the whole study group, while the 34 children who were not infected or were infected after the age of 2, experienced 40% of the lesions during the study period.[13] Therefore, both early colonization and high levels of salivary mutans streptococci are positively correlated with caries risk and early childhood caries.

The identification and quantification of *S. mutans* and *S. sobrinus* is part of an individual's caries risk assessment. Caries risk assessment is the determination of the likelihood of the risk of appearance of carious lesions during a certain time period.

2.3 Traditional Bacterial Culture

Traditionally, the most commonly used approach for enumeration of MS is selective culture. In the past few decades, the two most widely used selective media for isolating MS are mitis-salivarius-sucrose-bacitracin (MSSB) agar[15] and tryptone-yeast extract-cystine-sucrose-bacitracin (TYCSB) agar[16]. MSSB agar contains 0.2 units/ml bacitracin and 20% sucrose which generally allow growth of mutans streptococci while suppressing the growth of other oral streptoccocci including other oral streptococci species such as *Streptococcus salivarius*, *Streptococcus sanguinis* and *Streptococcus mitis*. However, oral bacteria other than MS can also grow on MSSB, which leads to the need for trained personnel to identify MS under the dissecting microscope, based on their unique colony morphologies. Further, the bacitracin and sucrose components in MSSB have some degree of inhibitory effect on growth of some mutans streptococci, which may result in under-enumeration or false negative reading of MS.[17, 18] In addition, bacterial culture is time and environments sensitive. The samples need to be transported

on ice and processed within 24 hours. This adds to the difficulty of using culture methods for caries risk assessment in routine clinical practice.

The reliability of MSSB agar and TYCSB agar in the selective culture of MS has been widely documented.[15, 18, 19] In fact, some studies reported a higher recovery rate of MS with TYCSB agar than with MSSB agar, with one study demonstrating the MS counts on the TYCSB agar to be higher by a factor of 10.[17, 18] While the recovery of MS appears to be higher with TYCSB agar, the disadvantages are the complexity of the TYCSB composition and difficulty preparing the media. MSSB media is easier to prepare and yields sufficiently optimal and selective growth of MS.

In summary, although selective culture has been a reliable method for MS quantification in caries risk assessment, there are a few major disadvantages. First, there is some degree of mutans streptococci inhibition by bacitracin and sucrose, resulting in decreased sensitivity or false negative results. [18] Second, culture requires phenotypic differentiation of bacterial colony morphology under a dissecting microscope by a trained microbiologist, which is time-consuming, laborious and not infallible. Third, species differentiation of similar-appearing bacterial colonies requires subsequent biochemical tests and thus more time and labor. Forth, maintaining bacterial vitality before plating is essential for selective culture. This requires storage under ice and rapid transport of saliva samples, requiring biohazard packaging and labeling, for microbiological assays within 24-48 hours of collection, which is inconvenient, expensive and generally not practical for private practice. All these barriers illuminate the need for a more accurate, efficient and convenient approach to MS identification and enumeration.

2.4 DNA-Based Methods

Molecular chromosomal DNA analysis has become an increasingly popular approach for the rapid and convenient identification and quantification of a wide array of microorganisms. Microbes ranging from periodontal pathogens such as *Porphyromonas gingivalis* and *Prevotella intermedia*[20] to viruses such as foot-and-mouth disease virus[21] have been detected and quantified via DNA-based techniques. The main advantage of DNA-based techniques is high specificity due to the utilization of nucleotide specific primers designed to target widely conserved chromosomal DNA sequences of the microorganism. DNA extracted from bacteria in saliva is more stable over time, allowing greater flexibility in handling in comparison to the immediate transport and processing of vital bacteria for selective culture. Where culture and biochemical tests may take up to a week, DNA-based methods can identify and quantify the desired product(s) in a few hours, saving time, labor and resources.

2.5 DNA Purification and Storage

A variety of methods of bacterial DNA extraction and storage in preparation for DNA analysis have been traditionally employed. The main challenge of DNA extraction for mutans streptococci lies in the ability to completely lyse the tough peptidoglycan cell walls of gram positive bacteria in order to obtain an optimal DNA yield for quantification. In 2000, a study extracted bacterial DNA from collected whole saliva by centrifuging for 15 minutes, adding lysis solution to the precipitate (10 mM Tris-HCl buffer, 1 mM EDTA, 1% Triton X-100, pH 8.0), vortexing, boiling for 10 minutes and collecting the supernatant which constituted the bacterial DNA.[22] In another study, researchers used lysozyme-coupled acid-washed glass beads to isolate DNA.[23] In these processes of DNA extraction, some DNA may be lost due to inability to lyse the cell wall and denaturing of DNA from high temperatures, toxic chemicals and/or unfavorable conditions.

A novel and innovative technique of nucleic acid collection, storage and purification is FTA Cards (Whatman Ltd., USA). FTA cards contain proprietary reagents that lyse cells upon contact and store nucleic acids in the fibers of the matrix at room temperature for an indefinite time period.[24] They contain chemicals that denature proteins and protect nucleic acids from nucleases, oxidative and ultraviolet damage.[24] This makes way for convenient handling, archiving and shipping of biological samples. FTA technology allows the purification of nucleic acids in preparation for PCR analysis in as little as 30 minutes. Genomic DNA stored on FTA cards at room temperature for over 17 years (and counting) has been successfully amplified by Polymerase Chain Reaction (PCR).[24] Another study has effectively PCR-amplified 100 samples of bacteria archived on FTA cards for over 3 years.[25] A goal of the present study is to assess the potential use of FTA technology for collection of saliva samples in private clinical practice, followed by subsequent qPCR analysis to determine bacterial challenge as a component of caries risk assessment.

2.6 Polymerase Chain Reaction (PCR)

(A) Introduction

PCR is a semi-quantitative technique that detects and amplifies the chromosomal DNA target exponentially in hours. PCR amplification requires a pair of specifically designed forward and reverse primers to bind to widely conserved regions of chromosomal DNA. The reaction, involving the DNA, primers, DNA polymerase, nucleotides and other necessary reagents, is performed in a thermocycler for replication. The target DNA undergoes amplification in three consecutive phases: Exponential, Linear and Plateau. The Exponential Phase in the earlier cycles of the reaction yields precise doubling of the product and highest efficiency. In the Linear Phase some of the reagents are consumed and efficiency decreases, leading to variability in the

amount of detected product. High variability in the amount of detected product results in the end-point Plateau Phase where amplification ends and products may degrade with time. In traditional PCR, the product is usually detected at the highly variable Plateau Phase. The results of PCR amplification are subsequently examined by electrophoresis in agarose gel. DNA is stained with ethidium bromide and visualized under short-wavelength ultraviolet light. The generated DNA ultraviolet band is compared with a standard DNA ladder to determine the base-pair (bp) size. A match of the bp size of the generated product with that of the expected product infers that the PCR assay successfully generated the desired product, however DNA sequencing is warranted for identity confirmation.

(B) DNA Probe and Primer Development for PCR Detection

The quest for the identification and detection of *S. mutans* via DNA-based methods has resulted in the development of a number of DNA-based probes and primers. Most of the primers have been designed to target specific sequences of genes associated with virulence, such as glucosyltransferases or dextranases, or highly conserved sequences of bacterial 16S rRNA genes.

In 1996 Igarashi *et al*. utilized primers formulated from the dextranase (*dexA*) gene for the detection of *S. mutans* by PCR.[26] Dextranase is an enzyme that hydrolyses glucans in plaque and is involved in the virulence of *S. mutans*. The method detected *S. mutans* (serotypes c, e, f) but none of the 16 strains of 12 other oral streptococcal species including *S. sobrinus*, *S. cricetus* and *S. downei*. The lower limit of detection was 12 CFU/ml. Seventy clinical isolates of *S. mutans* isolated from the dental plaque of 8 patients were all positive for *S. mutans*. This *dexA* PCR technique was successful in the specific detection and identification of *S. mutans*. [23]

A popular target for the development of primers for DNA-based detection, identification and quantification of MS has been glucosyltransferases genes that encode enzymes to produce

glucans which contribute to the formation of cariogenic dental plaque.[23, 27] Colby *et al*. in 1995 developed DNA probes for colony hybridization and PCR to detect *wapA* and *gtfA* genes in *S. mutans*, and successfully confirmed the absence of the *gtfA* gene in melibiose-negative isolates.[27] In 2000 Oho *et al*. used primers to target the *gtfB* gene of *S. mutans* and *gtfI* gene of *S. sobrinus* and both streptococcal species were detected and identified in clinical saliva samples.[22]

Other studies have identified and detected *S. mutans* via primer sequences from the 16S rRNA genes, which is highly conserved within bacterial species and is widely used as a tool of bacterial taxonomy.[28] In 2003 Sato *et al*. employed nested PCR for the detection of *S. mutans* and *S. sobrinus* in 18 dental plaque samples.[29] Nested PCR may increase detection sensitivity whereas direct PCR may have potentially low sensitivity for detecting bacteria from clinical samples. They first used universal primers 8UA and 1492R for the amplification of 16S rRNA genes, then conducted a second amplification of the first mixture with species-specific primers, sm1 and sm2 for *S. mutans* and SobF and SobR for *S. sobrinus*. The detection rate for nested PCR was 100% for *S. mutans* and 22% for *S. sobrinus*, which is more sensitive than direct PCR (44% and 11% respectively). The lower detection limit of *S. mutans* was 100 fg DNA.

(C) PCR Amplification in Reverse Capture to Quantify MS

Traditional PCR in combination with DNA hybridization techniques has been used to detect and quantify bacterial colonization levels. The sample bacterial chromosomal DNA, having undergone PCR amplification, hybridizes with strain-specific fluorescently-labeled probes cross-linked to a membrane via ultraviolet irradiation.

In this regard, a PCR-based, reverse capture checkerboard hybridization method has been employed with 16S ribosomal DNA sequence-based clonal analysis to detect and enumerate 23 known oral bacterial species, including *S. mutans* and *S. sobrinus*.[30] Becker *et al*. compared

bacterial levels in the dental plaque of 30 subjects ages 2-8 with severe caries (involving at least six teeth) with an age- and sex-matched healthy control group of 30 subjects. Bacterial levels in healthy subjects and at healthy sites in subjects with caries were compared for all species. Significantly higher levels of *S. mutans* were observed in subjects with caries than in healthy subjects. Within subjects with caries, bacterial levels found on intact enamel were compared to those found at each of the caries sites (white spot, cavitated lesions, and excavated carious dentin). A strong relationship to caries was observed for *S. mutans* at all lesion depths, while *S. sobrinus* was not found to play a major role in ECC. *S. sobrinus* has traditionally been difficult to detect in both culture and DNA-based methods, as a study suggested the detection level to be 11%.[29] Hybridization confirmed high levels and caries association of bacterial species *S. mutans*, *S. salivarius* and *Bifidobacterium*. The lower limit of detection for the assay was 10^5 cells/ml.

(D) PCR Limitations

While PCR exerts the advantages of greater specificity, convenience and fast delivery as compared to microbial culture, some major limitations are: 1) difficulty in designing species-specific primers due to intra-species gene variation that do not limit the detection of applicable strains; 2) semi-quantitative; 3) extra steps of agarose gel electrophoresis and DNA sequencing; and 4) extra steps of DNA extraction and preparation. PCR quantifies DNA from both dead and live bacteria, which may result in an increased count that is not clinically relevant because dead cariogenic bacteria in the oral cavity cannot produce acids that promote dissolution of tooth structure and therefore does not increase caries risk. Also, PCR detection sensitivity is moderate, as the lower limit of detection has been 10^4 CFU/ml in some studies [29, 31]. This means bacterial DNA concentration < 10^4 CFU/ml cannot be detected, rendering the absence or presence of

bacteria to be unknown. PCR data is collected at the end-point Plateau Phase of amplification where there is high variability and products may degrade with time, naturally resulting in decreased sensitivity.

2.7 *q*PCR

(A) Introduction

In recent years, quantitative real-time PCR (*q*PCR) has emerged as a novel, rapid and sensitive DNA quantitative technique. In *q*PCR the amplified DNA is detected and measured via fluorescence as the reaction progresses in *real time* or during each cycle, compared to standard PCR, where the product of the reaction is detected at its end. A major advantage of both *q*PCR and PCR is high specificity due to the utilization of nucleotide-specific primers designed to target widely conserved DNA regions. Where culture and biochemical tests may take up to a week, both *q*PCR and PCR can amplify the product in a few hours. The difference between *q*PCR and PCR is that *q*PCR enables higher sensitivity due to direct quantification of the copies of the target DNA sequence when normalized to a known standard. In addition, *q*PCR does not require the extra step of agarose gel electrophoresis, saving time and labor. Finally, FTA technology has allowed fast and easy storage and transportation of DNA templates for genetic assays.[25, 32] Easy sample handling is a benefit for large-scale screening and private clinical settings.

(B) TaqMan® vs. SYBR® Green

Two common methods for DNA detection in *q*PCR are: 1) non-specific fluorescent dyes that intercalate with any double-stranded DNA (i.e. SYBR® Green), and 2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which

permits detection only after hybridization of the probe with its complementary DNA target (i.e. TaqMan®). Fluorescence is detected and measured in the *q*PCR thermocycler.

There are advantages and disadvantages to both detection techniques. TaqMan® requires specific hybridization between the probe and target which increases specificity, however a different probe must be synthesized for each unique target sequence, rendering the TaqMan® assay relatively more expensive. SYBR® Green dye binds to any double-stranded DNA, thus specificity is highly dependent on the specificity of the primers as false positive signals may be generated if non-specific products are amplified. However, while TaqMan® is highly specific, it may limit detection of some MS strains since *S. mutans* comprise of a diverse group of oral streptococci strains. If specific primers are designed, *q*PCR with SYBR® Green will be sufficient to detect *S. mutans* strains in the oral cavity. In addition, each amplification product will have its unique melting temperature and melting curve (dissociation curve) of the *q*PCR product, so the specificity of the assay can be determined.

(C) SYBR® Green *q*PCR Assays

Two studies have documented the success of SYBR® Green *q*PCR in the detection and quantification of oral bacteria.

Yano *et al*. in 2002 established SYBR® Green *q*PCR assays targeting *gtf* genes of *S. mutans* and *S. sobrinus* for quantification in saliva samples from elementary school students.[23] Primers F5 and R4 were designed to anneal to conserved regions of *gtfB* and *gtfC* genes of *S. mutans* and amplify 415 bp DNA fragments from both genes. Primers F3 and R1 were designed to target conserved regions of *gtfI* genes of *S. sobrinus* and amplify 329 bp DNA fragments. The saliva samples were cultured and enumerated on both MSSB agar plates and modified MSSB agar plates and tested for biochemical characteristics. DNA isolation was done by lysozyme-

coupled acid-washed glass beads. Specificity was tested and confirmed using 31 different streptococci reference strains. *q*PCR assays were run in triplicates and the data was compared with culture via Spearman rank correlation coefficients. The results for *S. mutans* indicated a strong correlation between the *q*PCR assays and both the modified MSSB culture ($r=0.99$, $P<0.01$) and MSSB cultures ($r=0.96$, $P<0.01$). Likewise, for *S. sobrinus* there was also a strong correlation between *q*PCR assays and both modified MSSB ($r=0.97$, $P<0.01$) or MSSB cultures ($r=0.97$, $P<0.01$). The lower detection limit for *q*PCR was 10^2 CFU/ml. Although the authors identified a compelling correlation between *q*PCR and MSSB culture for quantification of *S. mutans* and *S. sobrinus*, they proposed some possible limitations: 1) cross reaction of the primers with *S. downei*, originally identified in macaque monkeys; and 2) difficulty in designing species-specific primers common to all strains due to intra-species variation of *gtfB*, *gtfC* and *gtfI* genes. Further study is warranted to develop a *q*PCR assay that has good adaptability to clinical practice and sensitivity for *S. mutans* quantification.

In 2007 Chen *et al*. used primer pair Sm479F/R derived from a 14-kb *Hae*III restriction fragment from *S. mutans* chromosomal DNA fingerprints for detection of *S. mutans* in mixed bacterial samples in SYBR® Green *q*PCR assays.[33] The primers were validated with 7 *S. mutans* reference strains, 48 non-ATCC *S. mutans* strains, 92 *S. mutans* clinical isolates, mixed DNA samples of *S. mutans* & *S. sobrinus* or *S. mutans* & *S. sanguinis*, and mixed bacterial DNA of saliva samples from thirty-three 18-month-old children. The results were that all of the *S. mutans* samples tested positive while none of the other streptococcal species were detected. The lowest DNA detection level was 10^3 copies. This demonstrated that the primer pair used was specific and sensitive enough for the detection of *S. mutans* in mixed bacterial samples.

(D) Taqman® *q*PCR Assays

In 2004 Suzuki *et al*. developed a TaqMan® *q*PCR assay for quantifying early colonizer microorganisms in dental biofilms.[34] Researchers used subtractive hybridization to prepare species-specific primers and TaqMan® probes for *S. gordonii, S. mitis, A. naeslundii,and A. viscosus*. The numbers of CFU were determined by plating culture dilutions on agar plates. They quantified the four oral bacterial colonizers and two cariogenic bacteria, *S. mutans* and *S. sobrinus*, in dental plaque which were collected from five individuals. Large numbers of *S. mitis* ($1.47x10^3$ to $1.96x10^5$ CFU/ml) were detected, while *S. gordonii* and *A. viscosus* had little or no detection. Low to moderate levels of *S. mutans* ($1.52x10^0$ to $1.25x10^4$ CFU/ml) and *S. sobrinus* ($2.02x10^0$ to $9.19x10^4$ CFU/ml) were detected. This study shows that TaqMan® *q*PCR combined with subtractive hybridization can be used for identifying and quantifying oral bacterial species in dental biofilm.

In 2007 Price *et al*. used TaqMan® *q*PCR to identify and quantify *S. mutans* and *S. sobrinus* using specific 16S rDNA primers.[35] The widely conserved genomic ribosomal Intergenic Spacer Region (ISR) that lies between the 16S and the 23S RNA genes has been used as a DNA target for bacterial identification and quantification. The authors collected saliva samples from 9 volunteers at the Walter Reed Army Institute of Research. The primer specificity was analyzed against lab reference strains of 15 other oral bacteria. They reported successful identification of *S. mutans* and *S. sobrinus* in clinical saliva samples and *in vitro* biofilms developed from clinical saliva samples. However, there was no culture or other microbiological test used to confirm the accuracy of the *q*PCR assays.

(E) Gene Regions and Primers Specific for *S. mutans*

The detection and quantification of specific oral streptococci species by *q*PCR involves the development of primers and probes specific enough to anneal exclusively to highly

conserved gene regions. The challenge is the development of species-specific primers that do not limit the strains detected due to intra-species gene variation.

As described above several research groups have designed primers pairs for the detection & enumeration of MS using conventional & *q*PCR methodologies. Many of these primer pairs were designed based on gene sequences found in MS glucosyltransferase (GTF) genes, from partial GTF gene sequences, and the fully sequenced *S. mutans* UA 159 strain.[36] Although most of these primer sequences have been shown to be specific for MS their degree of conservation amongst MS strains has not been rigorously tested or proven. Furthermore, most previous studies have not correlated their PCR results with culture using clinical samples to validate their methodologies.

Primer pairs derived from highly conserved 16S rRNA sequences of MS have also been reported. Primer pairs developed from these gene regions should demonstrate substantial specificity and would be highly conserved amongst MS strains. Therefore, we initially attempted to use the 16S rRNA MS primer pairs reported by Price *et al*. [35] in our studies reported below, however we were unable to produce PCR products with these primers and chose to utilize primers developed in another on-going study within our lab group (Zhan *et al*., unpublished data).

The rationale for primer development was that the sequences should be specific for MS and widely conserved amongst MS strains. Rather than using the standard approach of deriving specific primer sequences from known MS DNA sequences, an intuitive approach was employed. The general strategy was to use AP-PCR to recognized amplified DNA fragments common to a large number of stock culture and clinical isolates of MS and then to sequence those common products and derive specific primer pairs. Using this approach, forward and

reverse primers MSB2-1 and MSB2-R503 were derived from a common sequence in *S. mutans* species identified by AP-PCR. The specificity of the primers to *S. mutans* was confirmed by PCR assays: 7 *S. mutans* reference strains and 5 *S. mutans* clinical isolates tested positive; while 3 *S. sobrinus* strains, 1 *S. salivarius* strain, 1 *S. gordonii* strain, 4 *S. sanguinis* strains, 1 *S. mitis* strain, and 1 *S. milleri* strain were negative. A GenBank search of the primer sequences indicated 100% match with sequences from *S. mutans* UA159. The sequence has partial homology to a *Chlamydia* DNA sequence, however *Chlamydia* has not been shown to be a likely colonizer of the oral cavity. Therefore, we concluded the primer pair met the specificity requirements for use in *q*PCR assays.

3. AIM AND CLINICAL SIGNIFICANCE

The aims of this study were to 1) optimize and evaluate the efficacy of a SYBR® Green *q*PCR method using *S. mutans* specific primers by comparison to mutans streptococci culture, and 2) establish a correlation between *q*PCR enumeration of *S. mutans* and caries (DMFS/DS) scores in 60 clinical samples. While previous studies have successfully detected and quantified *S. mutans* and other cariogenic oral bacteria via *q*PCR, they have not assessed the clinical relevance of *q*PCR enumeration to caries risk assessment. A reliable *q*PCR method coupled with FTA technology that provides fast and convenient quantification of *S. mutans* in clinical saliva samples could facilitate the availability of *S. mutans* quantification to private practitioners as a component of caries risk assessment and caries prevention.

4. METHODS

4.1 Subject Recruitment and Sample Collection

Patient recruitment and sample collection was approved by the UCSF Committee on Human Research (CHR). The saliva samples analyzed in the present study by *q*PCR had been archived from two previous studies. Zhan *et al*. recruited 20 children aged 6-10 (10 males and 10 females) with at least 1 carious lesion from the UCSF Pediatric Dentistry clinic (unpublished). Informed consent was obtained for the collection of 2 saliva samples using different sampling methods: 1) saliva from chewing on parafilm and expectorating into a sterile tube; and 2) swipe of teeth, mucosa and gingiva with sterile cotton swab which was placed in a sterile test tube with 2 ml PBS. In another study by Zhan *et al*., 20 caries-free and 20 caries-active children aged 2-5 were recruited at the UCSF Pediatric Dentistry clinic (unpublished). The inclusion criteria were no clinically detectable caries and no restorations in the past 2 years. Saliva samples were collected with cotton swabs as described above for all caries-free children.

All samples were kept on ice and transferred to the microbiology lab for selective culture on MSSB agar within 24-48 hours of collection. The saliva samples were also archived at that time on FTA Cards, dried overnight, and stored at room temperature for subsequent *q*PCR assays. One-tenth ml of each clinical sample was applied onto a FTA matrix card with a micropipette in a circular movement from the center to the border of the sample area.

4.2 DMFS/dmfs and DS/ds Indices

A dental examination was done for all 60 subjects with DMFS/dmfs and DS/ds scores (decayed, missing (due to caries) or filled tooth surfaces) recorded. DMFS/dmfs and DS/ds indices have been traditionally used to determine the positive relationship of past caries experience, a significant factor in the assessment of caries risk, to mutans streptococci levels.[13, 37]

The principal investigator A.C. was blinded to the identity of the subject group, previous mutans streptococci culture counts and DMFS/dmfs/DS/ds scores.

4.3 Selection of Primer Pair for *q*PCR for *S. mutans*

In an unpublished study, Zhan *et al*. developed forward primer MSB2-1 and reverse primer MSB2-R503 based on a common sequence identified in AP-PCR products from lab reference strains and clinical isolates of *S. mutans*. The brief description of the development and initial testing of these primers are described in Background section 2.7 E. A detailed report regarding the development of these primers will be presented elsewhere (Zhan *et al*., unpublished data). The predicted PCR product with these primers is 261 base pairs (bp) with a melting temperature of 87º C.

4.4 *S. mutans* DNA Standard Preparation

S. mutans ATCC 25175 were recovered, from -80C frozen stocks, on BHI agar incubated anaerobically for 48 hours. Colonies were then inoculated in 2 ml Trypticase Peptone Yeast-extracts broth (TPY) for anaerobic overnight culture, and 0.1 ml of the overnight culture was subsequently inoculated in 2 ml TPY for a second overnight culture. Then 10-fold serial dilutions (10^{-1} – 10^{-6}) of the overnight culture were made using PBS (pH 7.2). One-tenth ml of each dilution was blotted on FTA cards for use as DNA standard templates for *q*PCR. In addition, a viable count of *S. mutans* 25175 in the overnight broth was determined by culture of the serial dilutions on MSSB plates. The FTA blots with viable *S. mutans* levels ranging from 10^2 to 10^8 CFU/ml were used as standards for *q*PCR assays. Genomic DNA of *S. mutans* ATCC 25175 was also extracted using a Qiagen Mini DNA extraction kit (Qiagen, CA, USA) with lysozyme pre-treatment for 30 minutes per manufacture instruction. The concentration of DNA was measured by a NanoDrop Spectrometer (ThermoScientific, DE, USA). A known amount of the liquid DNA extracted from *S. mutans* 25175 (10^3 CFU/ml) served as an internal positive control.

4.5 DNA Purification on FTA Cards

Two-mm punch portions of the FTA cards archived with standards and clinical samples were removed and placed in wells of a 96-well microtiter plate. Each assay consisted of No Template Controls (NTC), internal positive controls with or without FTA card punches, standards of 10^2 to 10^6 CFU/ml of *S. mutans*, and clinical samples. Each well was washed with 200 ul FTA Purification Reagent (Whatman Bioscience, USA) for 5 minutes with gentle agitation by pipetting up and down. This was repeated twice for a total of 3 washes. Each well was then further washed 3 times with 200 µl TE buffer (0.01 mM Tris HCl/EDTA, pH 8.0). The punches were then completely dried at 56 °C for 15 minutes and ready for *q*PCR assays.

4.6 *q*PCR Assays

The reaction solution consisted of SYBR® Green 2X master mix (Applied BioScience, USA), the *S. mutans* specific primers, and autoclaved sterile de-ionized water. The master mix was vortexed before adding to each well of an ABI 96-well *q*PCR plate. The plate was centrifuged for 2 minutes to ensure the FTA punches settled at the bottom of each well. The assay was run in an ABI *q*PCR machine under the following conditions: 50 ºC 2 min, 95 ºC 10 min, 40 repetitions of 95 ºC 15 sec, 60 ºC 1 min, 74 ºC 1 min. The dissociation curve (melting curve) was recorded per assay.

4.7 Data Analysis

Data analysis was performed using Pearson correlation test. *S. mutans* quantification by *q*PCR was compared to enumeration of MS by culture. The results from the saliva and swab samples were individually compared with the patient's DMFS/DS scores.

5. RESULTS

5.1 *q*PCR Co-efficiency Test and Specificity

A total of 60 clinical samples were analyzed by *q*PCR (20 saliva samples and 40 swab samples). Each clinical sample was run in triplicates per assay and all *q*PCR assays were repeated 3 times.

Figure 1 illustrates the standard curve of a sample *q*PCR assay with serial dilutions of *S. mutans* ATCC 25175 archived on FTA. SM DNA was purified on FTA as described previously prior to the *q*PCR assay.

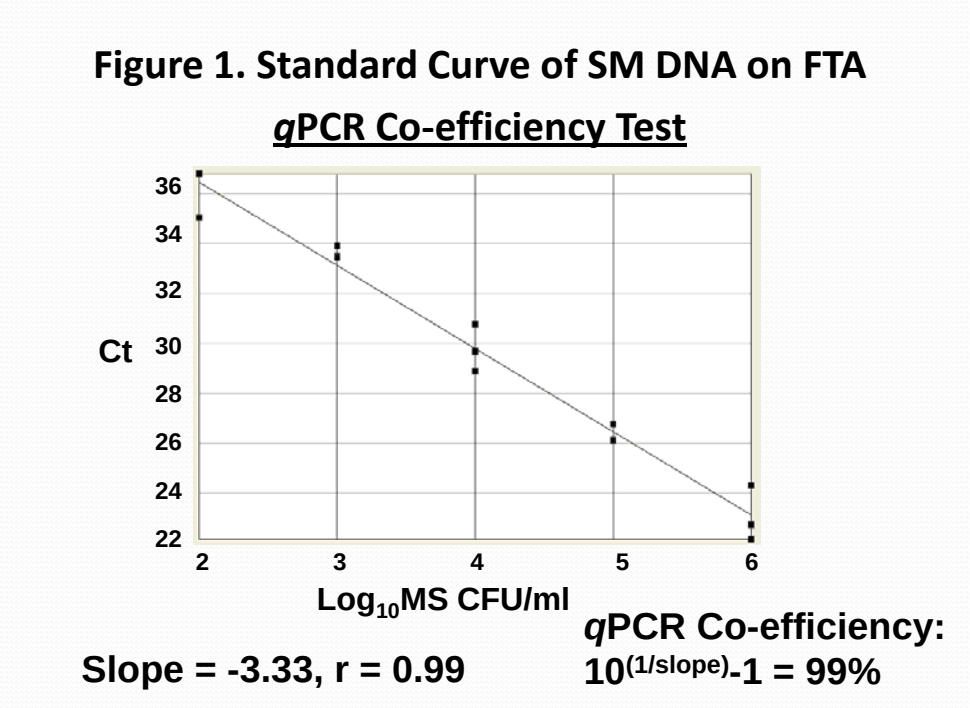

Figure 1. Sample *q*PCR assay demonstrating standard curve of *S. mutans* 25175 archived on FTA card (concentrations 10^2-10^6 CFU/ml). Co-efficiency = 99%, illustrating high accuracy and sensitivity of DNA amplification in the assay. The range of co-efficiencies for all *q*PCR assays was 85-99%.

The standard curve plots the logarithmic value of *S. mutans* counts (CFU/ml) enumerated by culture on the x-axis, versus the Ct value, the PCR cycle at which the fluorescence can be detected above threshold, on the y-axis. This standard curve, generated by serial dilutions of *S.*

mutans overnight culture with viable bacterial concentrations ranging from 10^2 to10^6 CFU/ml, was used to quantify *S. mutans* levels of clinical samples by *q*PCR. Using the standard curve, we calculated the co-efficiency of the *q*PCR assay. The co-efficiency is important for the accuracy and sensitivity of *q*PCR quantification. According to the *q*PCR co-efficiency, the slope for 100% *q*PCR co-efficiency is -3.2. The slope of the above standard curve was -3.33, which gives a *q*PCR co-efficiency of 99%. The linear regression tests showed a correlation co-efficiency r=0.99, illustrating a high correlation of viable standard culture counts of *S. mutans* to the Ct values. The range of co-efficiencies for all *q*PCR assays was 85-99%. The range of the linear correlation co-efficiency between MS viable culture counts and Ct for the standard curve was 0.87 to 0.99.

We also used the dissociation (melting) curve of the end product(s) of each *q*PCR assay to confirm specificity of the primers. A distinct melting temperature exists for each *q*PCR amplification end product. The melting temperature of our designated *q*PCR product for *S. mutans* is ~78 ºC. Figure 2 showed a typical melting curve at 78ºC for a *q*PCR assay of *S. mutans* in a clinical sample and verifies amplification of the desired product. The majority (55/60) of the dissociation curves verified the high specificity of the SYBR® Green *q*PCR assays for *S. mutans* detection. In our assays, 3 out of 60 tested samples had non-specific amplification products with wrong product melting temperatures (3 saliva caries-active samples). The levels of *S. mutans* in those three samples were recorded as 0 CFU/ml in the data analysis. Also, 2 caries-free swab samples had negative *q*PCR results.

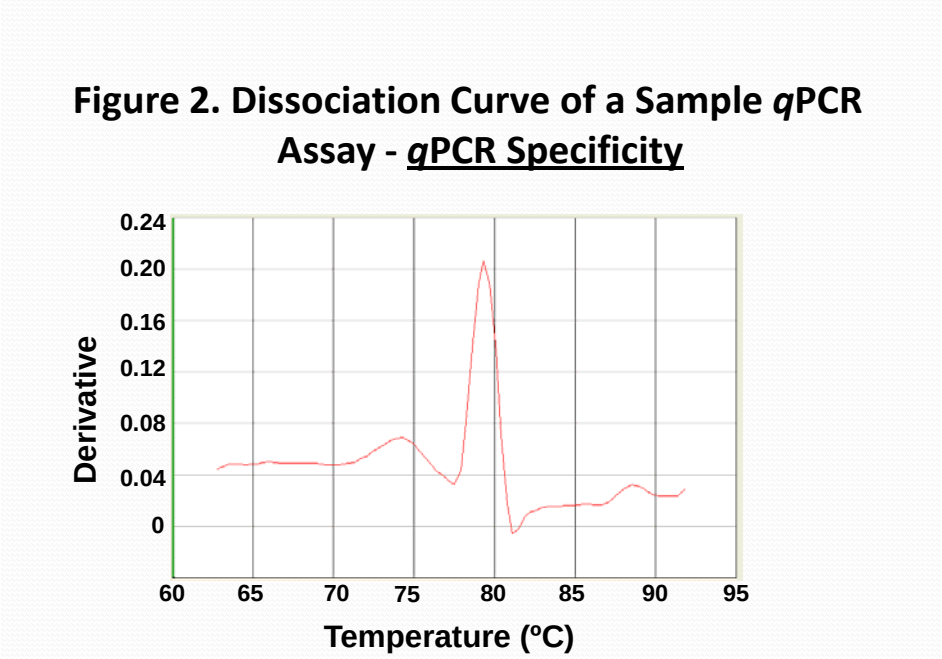

Figure 2. *q*PCR dissociation curve of a clinical sample demonstrating specificity of primers to the target DNA region in *S. mutans*. Fifty-five of 60 total samples analyzed provided *q*PCR products with correct melting temperature (87ºC). Three caries-active saliva samples had incorrect melting curves (non-specific amplification) and their values were recorded as 0. Two caries-free swab samples had 0 *q*PCR values

5.2. Correlation of *S. mutans* *q*PCR Quantification with MS Levels by Culture

Figure 3 demonstrated the correlation of *S. mutans* quantification by *q*PCR with MS enumeration by MSSB culture for the 60 clinical stimulated saliva and swab samples. *S. mutans* *q*PCR quantification had a moderate correlation with MS enumeration by culture using Pearson correlation test ($r=0.51$, $P<0.001$). Overall, more samples exhibited higher MS levels by culture than by *q*PCR (Fig. 3). From the linear regression shown in Figure 3, in general culture enumeration of MS was higher than *q*PCR quantification of *S. mutans* for counts $< 10^3$ CFU/ml, whereas the opposite was true for counts $>10^3$ CFU/ml. Sixteen samples (1 caries-active swab, 1 caries-active saliva, and 14 caries-free swab samples) had $\log_{10}MS = 0$ when enumerated by

MSSB culture. Of these 16 samples, the range of $\log_{10}$SM *q*PCR values was 0 to 6.04, with mean = 1.39 (generally low).

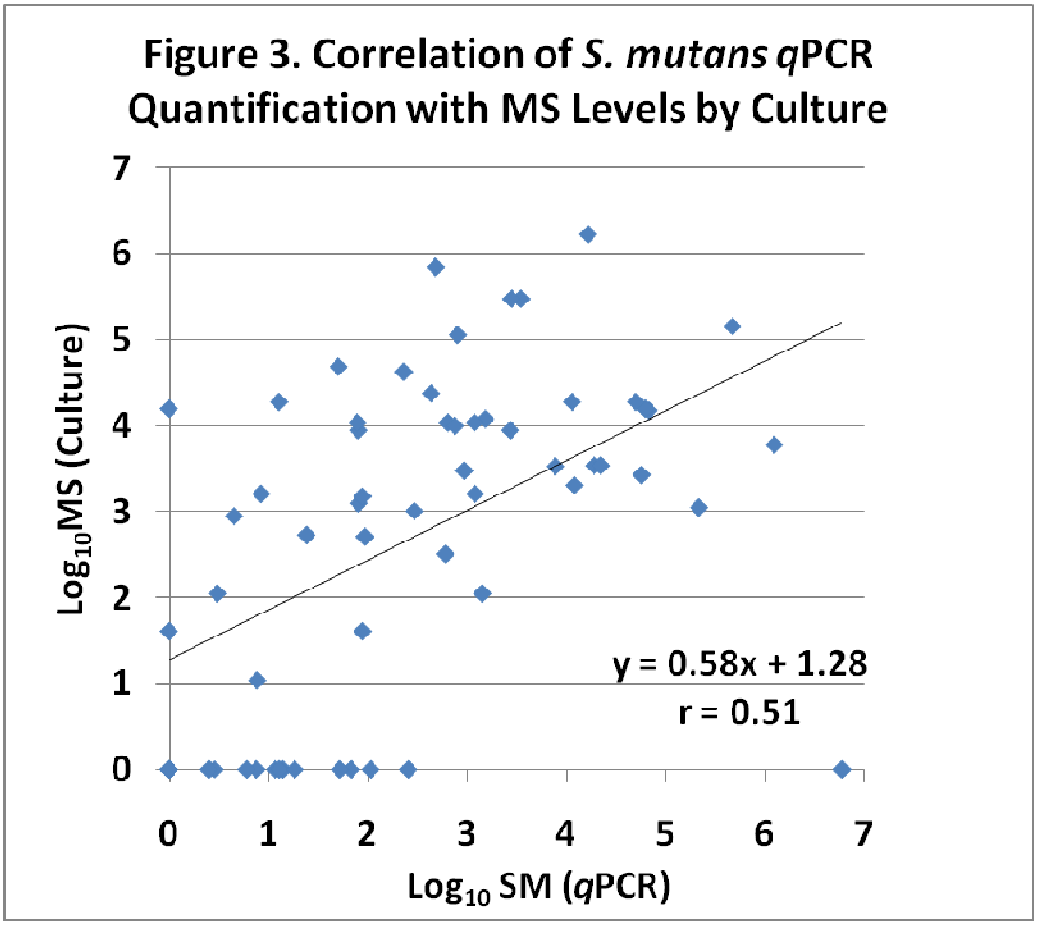

Figure 3. Moderate correlation of *S. mutans* *q*PCR quantification with MS levels by culture for the 60 clinical samples (r=0.51, P<0.001). Of the 16 samples where culture $\log_{10}$MS = 0, the range of $\log_{10}$SM *q*PCR values was 0 to 6.04 with mean = 1.39 (generally low).

Because of the high disagreement of MSSB culture and *q*PCR for the 16 clinical samples with culture $\log_{10}$MS = 0, we also analyzed the correlation of the two methods omitting these 16 clinical samples to see whether the correlation of the two assays would improve. Figure 4 compared *S. mutans* *q*PCR quantification with MS enumeration by culture for the 44 clinical samples where culture $\log_{10}$MS > 0. Pearson correlation test for analysis of the 44 samples yielded a lower correlation (r=0.40, P<0.001) than when the zero culture values were included

(Fig 3). The *q*PCR $\log_{10}$SM values for the 16 samples (Fig. 3) with culture $\log_{10}$MS = 0 were generally low (15 samples below $\log_{10}$SM = 2.5). This was near the limit of detection for the *q*PCR method as used in this study.

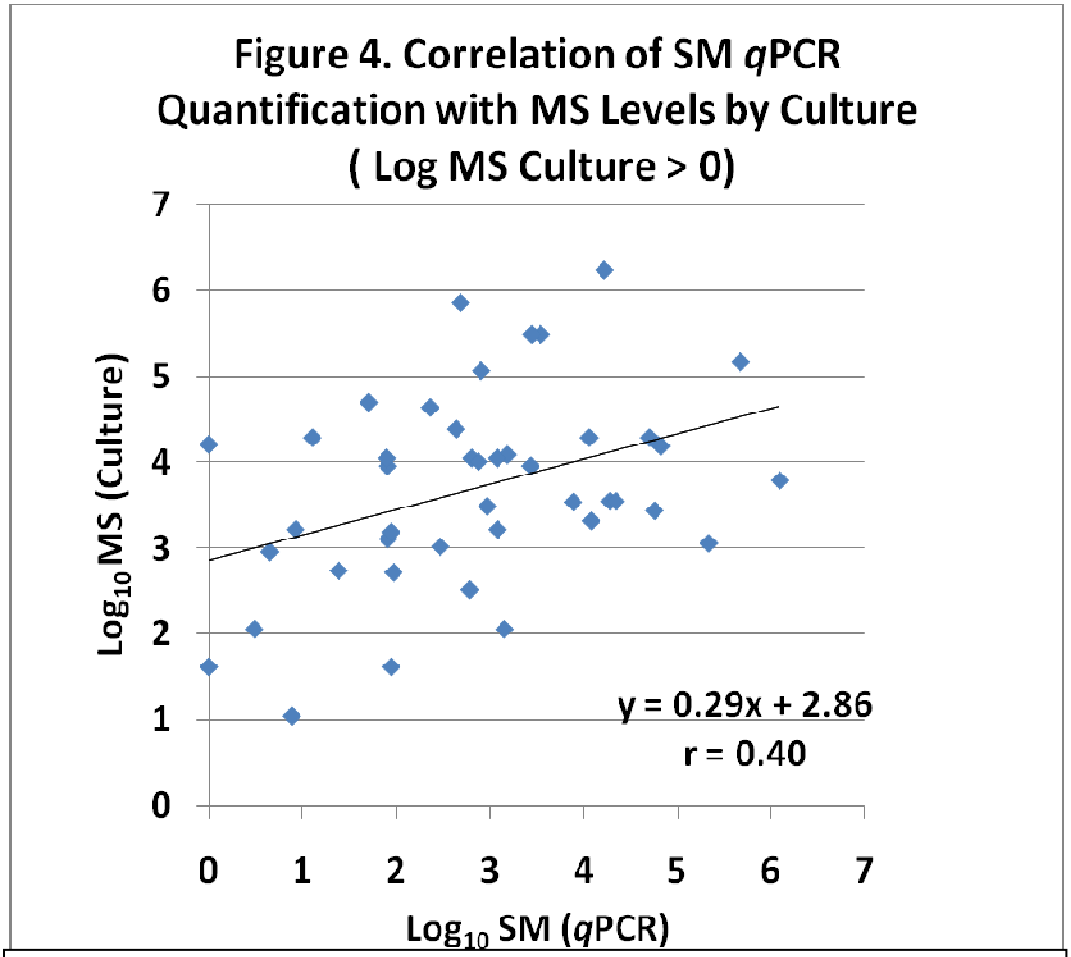

Figure 4. Correlation of *S. mutans* *q*PCR quantification with MS levels by culture (44 samples with culture $\log_{10}$MS > 0). Omitted 16 samples with culture $\log_{10}$MS = 0 to test for better correlation. The analysis yielded moderate correlation (r=0.40, P<0.001), lower than when the zero culture values were included in Figure 3.

5.3 Correlation of *S. mutans* *q*PCR Quantification with MS Culture - Saliva Samples

Comparison of *S. mutans* *q*PCR quantification with MS culture of the 20 saliva samples in caries-active children (Fig. 5) demonstrated a moderate correlation (Pearson correlation test,

r=0.52, P < 0.05). The linear regression in Figure 5 showed that, in general, culture had one to two times more counts than *q*PCR when values are < 10^3 CFU/ml. For the moderate and high range of caries risk (> 10^3 CFU/ml), culture and *q*PCR counts are more comparable. Three saliva samples with non-specific amplification for the product melting curves in the *q*PCR assays were recorded as 0 CFU/ml.

We also analyzed the correlation of culture logMS counts and *S. mutans* qPCR counts with patient's caries status. There was no significant correlation of *q*PCR or culture results with DMFS/dmfs or DS/ds scores (*q*PCR: r=-0.09, p=0.715 for DMFS and r=-0.24, p=0.311 for DS; culture: r=-1.91, p=0.419 for DMFS and r=-0.01, p=0.973 for DS).

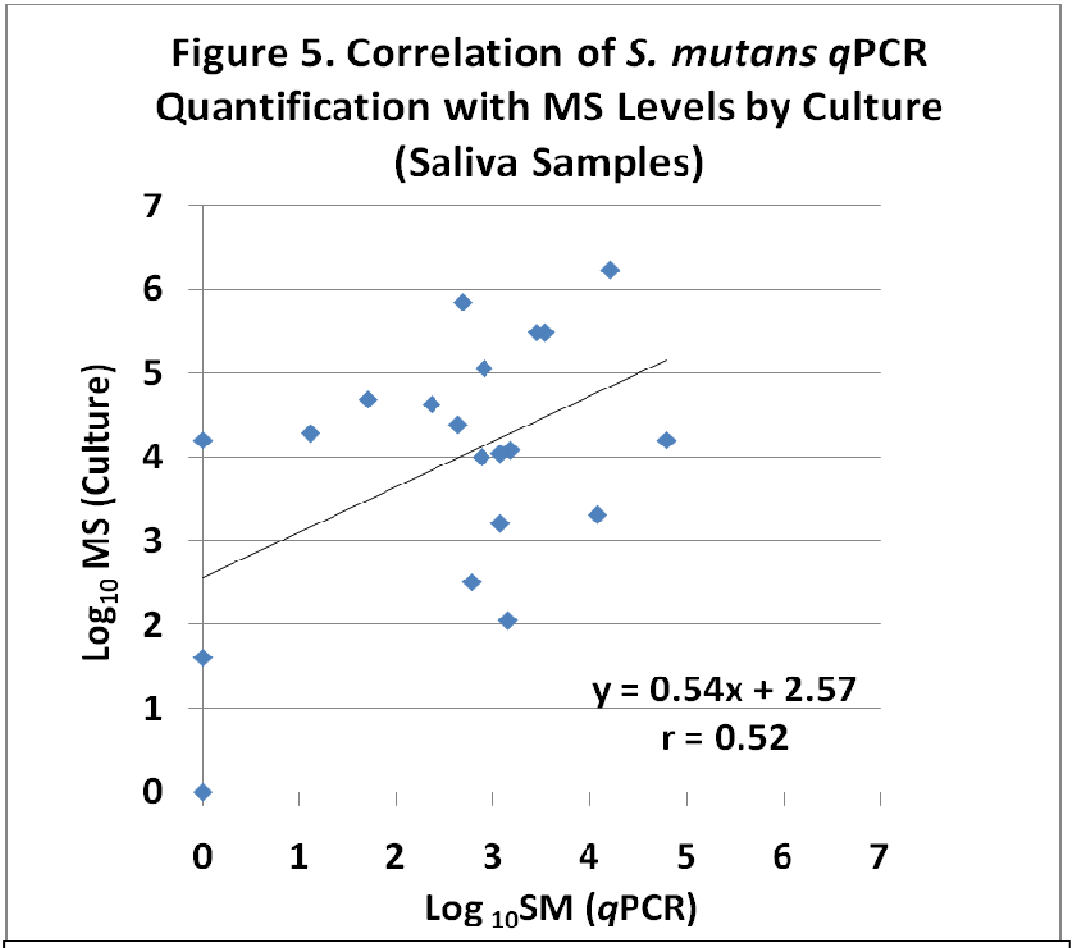

Figure 5. Moderate correlation of *S. mutans* *q*PCR quantification with MS levels by culture (20 saliva caries-active samples) r=0.52, P<0.05).

5.4 Correlation of *S. mutans* *q*PCR Quantification with MS Culture – Swab Samples

We also analyzed the correlation of bacterial counts enumerated by *q*PCR quantification for *S. mutans* and MSSB culture for total MS in the 40 swab samples from both caries-active and caries-free patients. The results (Fig. 6) illustrated a moderate correlation between the two enumeration methods (Pearson correlation test, r=0.51, P<0.001). Fifteen out of the 40 swab samples had culture $\log_{10}$MS = 0 (1 caries-active swab and 14 caries-free swab samples). There was not a significant correlation between the swab *q*PCR results and DMFS (r=0.17, p=0.305), but there was a significant correlation with DS (r=0.32, p<0.05). MS culture enumeration of the swab samples yielded a moderate correlation with DMFS (r=0.51, p<0.001) and DS (r=0.44, p<0.01) status.

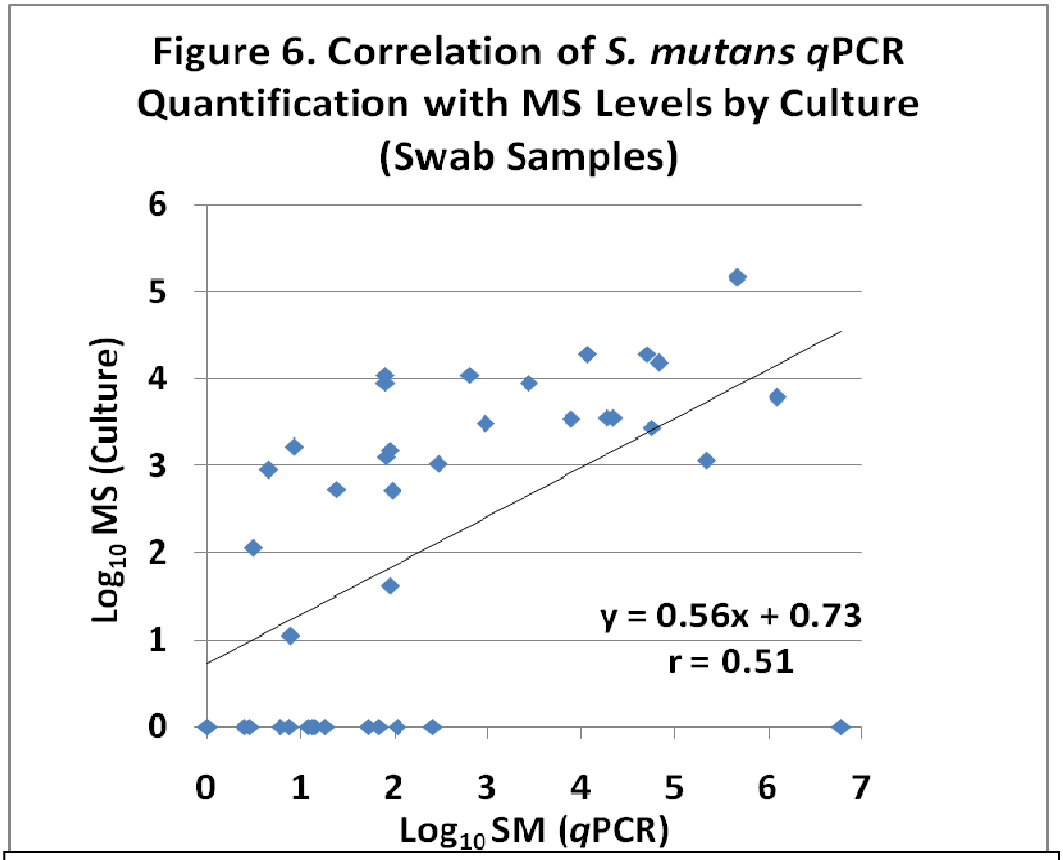

Figure 6. Moderate correlation of *S. mutans* *q*PCR quantification with MS levels by culture. Forty swab samples (20 caries-active and 20 caries-free), r=0.51, P<0.001

5.5 Reproducibility and Variance

The overall mean variance (CV) of all 60 clinical samples for *S. mutans* quantification by *q*PCR was 0.63 with SD=0.65 and range 0.05 to 3.00. We noticed that samples with *q*PCR quantification under the hypothetical detection sensitivity ($\log_{10}$MS < 3 CFU/ml) had larger variation compared to samples with readings above the detection limit. Therefore, the mean variance of *q*PCR assays in the clinical samples with *q*PCR $\log_{10}$SM > 3 CFU/ml was 0.13 with SD = 0.07 and range 0.05 to 0.26, demonstrating an acceptable variance and reproducibility.

6. DISCUSSION

A few previous studies have successfully employed *q*PCR in the convenient and rapid quantification of common oral cariogenic streptococci, namely *S. mutans* and *S. sobrinus*[23, 34, 35]. This study advances the utility of *q*PCR quantification of *S. mutans* by validating a simple reliable sample archiving technique for use in private clinical practice, and establishing a significant correlation of *S. mutans q*PCR enumeration and MS culture counts with caries (DMFS/DS) scores in clinical swab samples.

In general, we expected that MS levels determined by *q*PCR would be higher than culture since *q*PCR quantifies DNA from both live and dead bacteria, whereas only live bacteria would be detected by culture. Additionally, there is some inhibition of MS growth on MSSB agar and recovery has been shown to be about 46%[17]. Some MS strains may also not grow or be recognized on MSSB agar, but would be detected by *q*PCR assays. Sixteen of the 60 samples we analyzed were negative for MS by culture, but positive by *q*PCR. This suggests that the qPCR assay may have greater sensitivity in detecting *S. mutans* that could be inhibited by culture. It is interesting to find that 14 of these 16 samples with culture $\log_{10}$MS = 0 were from samples

obtained from caries-free subjects, who would be expected to have low levels of *S. mutans*. The *q*PCR results for these samples ranged from 0.18 to 6.04. However most (14/16) of these values (mean = 1.39 and median = 1.2) were in the range of low caries risk ($\leq \log_{10}$SM 3), and therefore very comparable to our culture results in assessment of bacterial challenge in regard to caries risk assessment.

Surprisingly we found only 19/60 (32%) of our samples had higher *q*PCR values than culture. One reason why some of the *q*PCR results were lower than culture may be that both *S. mutans* and *S. sobrinus* are grown and enumerated on MSSB culture, while the *q*PCR procedure used in the present study specifically detects and amplifies only *S. mutans*. Biochemical tests were not done to differentiate the two *Streptococcus* species. It is also possible that the primers used for *S. mutans q*PCR quantification may not completely encompass the total genetic diversity of *S. mutans* strains, thus limiting the quantity detected.

We assayed 60 clinical stimulated saliva and swab samples by *q*PCR to determine MS colonization levels. Dissociation curve (melting point) analysis of the *q*PCR products indicated non-specific amplification in only 3 of these samples. Fifty-five of 60 total samples analyzed provided *q*PCR products with correct melting temperature (87ºC), validating the specificity of the primers to *S. mutans* and the efficiency of the *q*PCR assays. Chen *et al*.[33] also utilized melting curves to verify product specificity when detecting *S. mutans* by *q*PCR in 14/33 (42.4%) saliva samples, as compared to 5/33 (15.2%) from culture, demonstrating significantly improved sensitivity with *q*PCR over culture.

We found a moderate correlation between *q*PCR quantification and *S. mutans* enumeration by traditional MSSB culture for the 60 clinical samples we assayed (a *q*PCR value of zero was assigned to 3 samples with non-specific DNA products). Overall, this demonstrates

that the SYBR® Green *q*PCR assay can be used as a reliable tool for rapid quantification of *S. mutans* without the inconvenience and labor of immediate transport and microbial culture.

A previous study by Yano *et al*.[23] has reported a stronger correlation between MS enumeration by *q*PCR and traditional culture (r=0.96-0.99) than we observed (r=0.51). Differences in assay sensitivity may have contributed to this discrepancy or *S. sobrinus* may also be less prevalent in that population. Yano *et al*.[23] reported the sensitivity of their *q*PCR assay to be 10^2 CFU/ml, whereas the sensitivity of our assay was 10^3 CFU/ml, similar to that reported by Chen *et al*.[33]

In order to determine if the 16 samples that were positive for MS by *q*PCR but negative by culture contributed to the comparatively lower correlation we observed compared to Yano *et al*., we analyzed the remaining 44 samples separately. However, compared to the investigation of all 60 samples, we found an even lower yet still moderate correlation (r=0.40, P<0.001) for the 44 clinical samples after omitting the 16 samples with culture $\log_{10}MS = 0$.

Since removal of samples with culture $\log_{10}MS = 0$ did not improve the correlation between *q*PCR and culture results we observed, we investigated the effect of sample collection technique on our results by analyzing stimulated saliva samples and swab samples separately. These separate analyses (stimulated saliva r=0.52; swab, r=0.51) also did not increase the correlation we observed with all samples, but indicate that both sampling procedure are applicable in future studies.

A primary goal of this study was to establish FTA technology and *q*PCR as a methodology for MS quantification that would potentially have utility in private practice for determination of bacterial challenge, a component of caries risk assessment. In this regard, in

addition to finding a moderate correlation between MS quantification by *q*PCR and culture, we investigated the correlation between our results and clinical caries measurements.

For the 40 swab samples from caries-free and caries-active children, we found significant correlations between SM levels by *q*PCR with DS status ($r = 0.32$, $P<0.05$) and MS levels by culture with DS ($r=0.44$, $P<0.01$) and DMFS ($r=0.51$, $P<0.001$). For the 20 saliva samples taken only from caries-active children we did not find a significant linear correlation between SM levels by *q*PCR or MS levels by culture with DS or DMFT, although most of these samples, as expected, exhibited moderate ($>10^3$ CFU/ml) to high MS levels by culture (16/20) and *q*PCR (10/20).

One limitation in this study is that we only detected and quantified *S. mutans*. Although *S. mutans* is the most common cariogenic bacteria isolated, other cariogenic bacteria such as *S. sobrinus* are instrumental in the initiation and progression of smooth surface caries; lactobacilli, known for their role in secondary caries formation, should also be taken into account as part of the total bacterial challenge for caries risk assessment.[5] This would require further study to develop specific primers and *q*PCR assays for enumeration of *S. sobrinus* and lactobacilli.

7. CONCLUSIONS

Quantification of salivary *S. mutans* levels from clinical saliva samples archived on FTA cards by SYBR® Green *q*PCR with primers MSB2-1 and MSB2-R503 cards demonstrated moderate to high efficiency, reproducibility and statistically significant correlation with traditional selective culture enumeration. *q*PCR coupled with FTA technology offers a fast and convenient quantification method for enumeration of *S. mutans* in clinical saliva samples applicable to private clinical practice for determination of bacterial challenge, a critical

component of caries risk assessment. Further studies extending to other cariogenic bacteria, namely *S. sobrinus* and lactobacilli, are needed for a more thorough evaluation of cariogenic bacterial challenge.

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