VARIABILITY OF TWO SAMPLING METHODS IN PLAQUE SAMPLES

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INTRODUCTION: Dental caries remain the most common infectious disease in children, and is in fact, on the rise in the U.S. among 2 to 5 year olds. Among hundreds of oral bacteria, mutans streptococci (MS) are considered the major species associated with dental caries. Studies have shown that increased MS in saliva or plaque is an important indicator for caries. The use of standard plate count (SPC) from plaque samples particularly yield variable quantitative results because of complexities of sample collection and processing.

OBJECTIVES: This study was undertaken to determine collection reproducibility in plaque samples from permanent first molars using two methods of sample collection. MATERIALS and METHODS: Plaque samples were collected from groups of healthy subjects, aged 17–50 and 6–12 years. Each subject was randomly assigned to either “I” (individual tooth collected-first sampling) or “P” (pooled teeth -first sampling) group. Plaque samples were processed and quantified for total streptococci (TS) and Streptococcus mutans (Sm) using traditional plate culture. The Sm/TS ratio was used to determine the reproducibility within “P” and “I” group samples. RESULTS: Mean percentage of Sm/TS in pooled and individual sampling methods were 1.64 and 1.21, respectively (not significantly different, p>0.05, t-test). However, for detection of Sm, there was a significant difference between pooled and individual sampling methods (individual-sampling more sensitive, p<0.05, McNemar’s test). CONCLUSIONS: Despite the lack of a significant difference between the sampling methods for quantitation of Sm/TS, the difference in Sm detection suggests that
individual samples (i.e. more samples collected) are more consistent; however the added resources requiring 4 times the number of samples to be processed might not be justified by the gain in sensitivity.
DEDICATION

TO MY PARENTS AND SISTERS: FOR YOUR GENEROUS LOVE AND ALWAYS BEING THERE FOR ME. WITHOUT YOUR FULL SUPPORT, I WOULDN’T BE ABLE TO GO THIS FAR.

TO MY FIANCÉ, RICKY: WITHOUT YOUR KINDNESS AND GREATEST PATIENT, I WOULD NOT BE ABLE TO GO THROUGH ALL THE PROCESS AND HANDLE THE STRESS COME AFTER.
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I WOULD LIKE TO THANK…….

MY MOST KNOWLEDGEABLE MENTORS, DR. CHILDERS AND DR. OSGOOD: YOU ARE THE GREATEST AND WONDERFUL MENTORS I HAVE EVER HAD. WITHOUT YOUR GUIDENCE AND TURORING, I WOULD NOT LEARN THIS MUCH AND FIND THE PATH TO MY CAREER.

MY OTHER COMMITTEE MEMBERS, DR. CUTTER, DR. RUBY, DR. THRONTON, DR. RAHEMTULLA: WITHOUT YOUR TRUTHFUL AND THOUGHTFUL PERSONALITY AND OPINIONS, I WOULDN’T BE ABLE TO FINISH THE STUDY AT UAB AND THIS THESIS.

ALL MY FRIENDS: BECAUSE OF YOUR FRIENDSHIP, I WOULD BE ABLE TO STUDYING HERE AT UAB. BITTER OR SWEET, I AM VERY HAPPY YOU ARE ALWAYS AROUND.
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INTRODUCTION

Dental caries is one of the most common chronic diseases in the U.S. even though it is preventable. According to the national survey by Center of Disease Control and Prevention (CDC), almost 60% of children suffer from tooth decay and nearly one in three adults have untreated cavities. Despite an overall decline in the total population, a latest survey in the U.S. shows an increase in caries rate in the age of 2~5 years from 1999 to 2004 (1). The high and increasing prevalence support the importance of dental caries prevention and lead to the significance of performing clinical caries risk assessment in order to facilitate clinicians to create a proper treatment plan and determine the level of prevention for each individual.

Etiology of Dental Caries

Dental caries is a chronic and infectious disease, which stems from multiple risk factors, including family history, persistence of dental plaque and excessive exposure to dietary sugars. In fact, the decay process does not occur in the absence of either dental plaque or fermentable dietary carbohydrates, making dental caries a dietobacterial disease (2). Among hundreds of bacterial species in the human oral cavity, mutans streptococcus (MS) has been implicated with a major etiological role in dental caries initiation and development based on overwhelming data since the 1960s (3). Tracing back to the history
of dental caries research, the focus has been targeted on oral bacteriology. In the late 19th century, Miller demonstrated the role of acid in the development of dental decay (4). He later called this, the chemoparasitic theory after incubating an extracted tooth with saliva and bread. He and his followers also attempted to correlate dental decay with specific bacterial species. However, he was unable to do so because most of the isolates he tested were capable of fermenting carbohydrates to acid. Therefore he concluded that the decay was due to collective acidogenic properties of plaque bacteria. In the early 20th century, acid tolerance was hypothesized to be a necessary property of bacteria relating to dental decay.

It was not until 1924 that, Clarke isolated Streptococcus mutans from an early carious lesion and showed that it can be isolated in higher proportion than lactobacilli (5). However, his work was not easy to duplicate because of the difficulty of distinguishing S. mutans from other oral streptococci due to the limitation of culture media available during that time. This situation caused other investigators to fail in their attempts to find S. mutans and this organism eventually became a non-entity. Later in mid-20th century, even though experiments with antibiotic treatments and germ-free animals proved the need for bacteria in the development of dental caries, S. mutans had not yet been discovered nor considered the primary agent for dental decay until the 1960s. New differential culture media became available to help researchers to rediscover S. mutans. Other animal experiments, associational and longitudinal studies, along with the rediscovery of S. mutans, lead caries researchers to the Specific Plaque Hypothesis. This hypothesis focuses on the change in the plaque ecology including elevation of S. mutans,
lactobacilli numbers and other acidogenic and acid tolerant species after frequent sucrose consumption.

This idea has been modified with the non-specific plaque hypothesis as the Ecological Plaque Hypothesis of the 21st century. The center point of this hypothesis is that an imbalance of acidogenic plaque bacteria can lead to dental caries, meaning that the ecological balance of the metabolic activities of all plaque inhabitants likely influences caries susceptibility. Furthermore, host factors likely affect the microbial ecological balance as well.

MS, as it relates to the pathogenesis of dental caries in humans, is composed of *Streptococcus mutans* and *Streptococcus sobrinus*. Previous research has identified the relationship between the activity of dental caries, the quantity of MS, and the timing of initial infection in the oral cavity (6). Among other plaque colonizers, MS are somewhat unique in their ability to metabolize sucrose, which allows it to flourish. In addition, because of its acidogenic and acidouric properties, it is thought to be specialized in the pathogenicity of dental caries. Once MS colonize teeth as part of the plaque building process, it adversely affects the enamel surface by demineralizing it with the acid byproducts of dietary sugar metabolism. Subsequently, tooth demineralization leads to caries (cavitation), suggesting that the quantity of MS in the saliva or dental plaque could be an important indicator for assessing caries risk (7, 8). Since *S. mutans* is most commonly isolated and associated with human samples, this species is the focus of most caries research. However, the most efficient means to collect a representative oral sample (i.e. saliva or plaque) and accurately quantify *S. mutans* remains an open question.
Sampling Methods for Oral Samples

Stimulated saliva and plaque are two of the most common oral samples collected for research and clinical tests. Although saliva is easy to collect and considered representative of colonized teeth, it poses two problems. First, there is no consistent and standard way to collect and process saliva for enumeration. Second, the volume, infusion of and duration of saliva contact as it interacts with enamel surfaces leads to variability and lack of reproducibility. Plaque samples, on the other hand, are direct samples of the bacteria colonizing teeth, including the MS, yet relatively complicated to collect, due to variability in efficiency of the amount collected and complexity in the number of teeth and the surfaces available for collection (9). In this regard, the nature of plaque, which is a dynamic accumulation of organisms on tooth surfaces, presents an interesting dilemma in the assessment of MS for caries risk. Two aspects of sampling may dramatically influence the accuracy of the observed counts. One is the location of the sample relative to colonization of the tooth and the second is the method of collection. As such, the collection issues associated with plaque are to some degree less complicated in a saliva sample. One must, however assume that saliva by virtue of its exposure to the tooth surfaces (bacteria sampling) is a homogeneous sample accurately represents to what is present at the dental surfaces (10). Therefore, this assumption is somewhat presumptuous and leads to a very general approximation that may not accurately characterize risk for dental caries. Due to the nature of plaque collection, studies that in require its use can yield conflicting information if these methods are not standardized as much as possible. There are some studies that have used plaque collected from individual teeth and other studies that have used pooled plaque samples from several teeth. Even assuming that the
collection procedures for both approaches have been standardized, it is only possible to make a qualitative comparison between the two collection procedures. Given the possibility of variation that may be seen from any given plaque sample, it is reasonable to contemplate the superiority of one collection method over the other as it pertains to the highly consistent detection of \textit{S. mutans}. Therefore, the hypothesis of this study is that a pooled plaque sample is representative of individual samples and provides a higher degree of detection of \textit{S. mutans} than individual samples collected from 4 permanent first molars.

In this study, we used the standard plate counting method to process plaque samples in order to determine the collection reproducibility of two collection methods: pooled sampling and individual samples from four permanent first molars. Hence, the specific aims for this study are: 1) to quantify \textit{S. mutans}/total streptococci of plaque samples using pooled and individual sampling methods from four permanent first molars for comparison of these two methods. 2) to compare reproducibility and sensitivity of detecting \textit{S. mutans} between individual sampling and pooled sampling methods. The purpose of this study is to determine which plaque collection procedure, pooled or individual, gives the best reproducibility and least variability in detecting \textit{S. mutans}, by blindly and randomly assigning all study subjects to either an individual “I” or a pooled “P” plaque collection group and to then subject all ten-fold serially diluted samples to standard plate counting on alternating duplicates of Gold’s and mitis salivarius (MS) agar to quantitate \textit{S. mutans} and total streptococci, respectively.
Variability of Two Sampling Methods in Plaque Samples

by

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Abstract

Introduction: Dental caries remain the most common infectious disease in children, and is in fact, on the rise in the U.S. among 2 to 5 year olds. Among hundreds of oral bacteria, mutans streptococci (MS) are considered the major species associated with dental caries. Studies have shown that increased MS in saliva or plaque is an important indicator for caries. The use of standard plate count (SPC) from plaque samples particularly yield variable quantitative results because of complexities of sample collection and processing.

Objective: This study was undertaken to determine collection reproducibility in plaque samples from permanent first molars using two methods of sample collection: a pooled sample and four individual tooth samples. Materials and Methods: Plaque samples were collected from two groups of healthy subjects, one aged 17–50 and the other 6–12 years. Each subject was randomly assigned to either “I” (individual tooth collected-first sampling) or “P” (pooled teeth-first sampling) group. Plaque samples were processed and quantified for total streptococci (TS) and Streptococcus mutans (Sm) using traditional plate culture method. The Sm/TS ratio was used to determine the reproducibility within “P” and “I” group samples. Results: Mean percentage of Sm/TS in pooled and individual sampling methods were 1.64 and 1.21, respectively (not significantly different, p>0.05, t-test). However, for detection of Sm, there was a significant difference between pooled and individual sampling methods (individual-sampling more sensitive, p<0.05, McNemar’s test). Conclusions: Despite the lack of a significant difference between the sampling methods for quantitation of Sm/TS, the difference in Sm detection suggests that individual samples (i.e. more samples collected) are more consistent; however the added resources requiring 4 times the number of samples to be processed might not be justified
by the gain in sensitivity.
Introduction

The latest data from the U.S. national survey indicates that while dental caries are decreasing overall, young children (2 to 5 years old) are experiencing increased prevalence (1). Dental caries is a chronic disease associated with multiple risk factors, including: dental plaque and excessive exposure to dietary sugars. It is proposed that the decay process does not occur in the absence of either dental plaque or fermentable dietary carbohydrates; thus, dental caries must be considered a dietobacterial disease (2). In fact, since dental caries is an infectious disease, it is the most prevalent disease known to humans. Among hundreds of bacterial species in the human oral cavity, overwhelming data supports colonization with mutans streptococci (MS) as a major etiological role in dental caries initiation and development (3). MS, as it relates to the pathogenesis of dental caries in humans, are composed of *Streptococcus mutans* and *Streptococcus sobrinus* (11, 12). Since *S. mutans* is most commonly isolated and associated with human samples, this species is the focus of most research (3). Many studies have identified the relationship between the activity of dental caries, the quantity of the MS, and the timing of initial infection in the oral cavity (2, 3, 6). Among other plaque colonizers, MS is somewhat unique in its ability to metabolize sucrose and because of its acidogenic and acidouric properties (3, 13, 14), it is thought to be specialized in the pathogenicity of dental caries. Once MS colonize teeth as part of the plaque building process, the tooth surface potentially comes under attack from the acid byproducts of dietary sugar metabolism predominantly from this organism. Subsequently, tooth demineralization leads to caries, an observation suggesting that the quantity of MS in the saliva or dental plaque could be an important indicator for assessing caries risk. Previous
studies have used either traditional plate culture methods or more recently PCR methods to detect and determine the quantity of *S. mutans* (15-17). Therefore, to evaluate caries risk, it is necessary to develop means to collect saliva and/or plaque samples to accurately and reliably quantify MS/ *S. mutans*.

Although saliva is easy to collect and considered representative of colonized teeth, it poses two problems. First, there is no consistent and standard way to collect and process saliva for enumeration. Second, the volume, infusion of and duration of saliva as it interacts with enamel surfaces leads to variability and lack of reproducibility. Plaque samples, on the other hand, are direct samples of MS containing substances, yet relatively complicated to collect, due to variability in efficiency of amount collected and complexity in the number of teeth and the surfaces available for collection (9). In this regard, the nature of plaque, which is a dynamic accumulation of organisms on tooth surfaces, presents an interesting dilemma in the measurement of MS for caries risk assessment. Two aspects of sampling may dramatically influence the accuracy of the observed counts. One is the location of the sample relative to colonization of the tooth and the second is the method of collection. As such, the collection issues associated with plaque are to some degree less complicated in a saliva sample. One must, however assume that saliva by virtue of its exposure to the tooth surfaces (bacteria sampling) to be a surrogate for what is present at the dental surfaces (10). Therefore, this assumption is presumptuous in that it may lead to a general approximation which may not accurately characterize risk.

The methods outlined above are criticized for lacking accuracy, sensitivity and precision that are necessary to satisfactorily quantify the MS/ *S. mutans*, as well as being
time-consuming and laborious. Nevertheless, traditional culture remains one of the most common methods of quantifying bacteria and this quantitative technology has been presented to clinical practice by the marketing of culture kits to assess caries risk, such as CRT (Vivadent, Amherst, NY), Saliva-Check (GC America Inc., Alsip, IL), and the Dentocult SM Strip mutans test (Orion Diagnostica, Espoo, Finland). The purpose of this study is to compare two different plaque collection methods (i.e., individually sampled index teeth vs. a pooled single sample collected from a number of index teeth) for quantifying *S. mutans* from permanent first molar teeth. The goal is to determine which method is most representative for reproducibly assessing plaque samples in a population to aid in the assessment of caries risk associated with *S. mutans* colonization.

**Materials and Methods**

**Experiment Design**

Seventeen adults (age 17 – 50 years old) and 18 children (age 6 – 12 years old) were recruited, consented for *S. mutans* and total streptococci sampling, and asked to complete a medical/dental history questionnaire. Inclusion criteria stipulated that subjects be healthy adults and children with all four permanent first molar teeth fully erupted and present in the oral cavity. In addition, subjects must not have taken antibiotics within two weeks prior to the sample collection. Dental exclusion criteria consisted of the presence of extensive dental caries, periodontal disease, or other obvious oral disease. Adults were recruited from the University of Alabama at Birmingham (UAB) employees and students by word of mouth. Children were recruited from patients in the UAB School of Dentistry. After explanations of planned procedures and the study’s risks/benefits, the subjects’
questions were answered, and informed consents were obtained. A visual dental examination was completed to determine eligibility, and then multiple plaque samples were collected from the four first permanent molars under the IRB approved protocol described below.

**Plaque collection methods and processing**

Subjects were randomly assigned to either the “I” group (four individual tooth samples collected first, then a pooled sample was collected) or the “P” group (pooled samples over the same four teeth collected first, then individual samples were collected) using previously prepared, sequentially numbered and sealed envelopes that contained the randomized assignment. Ten plaque samples (i.e., 2 sets of individual and 2 pooled samples) were collected from buccal/mesial/distal/lingual/occlusal surfaces of either individual permanent first molars (#3, #14, #19, #30) or pooled (all four) permanent molars using a sterile tooth pick and transferred to sterile saline as illustrated in Fig 1.

Plaque samples were sonicated for 20 seconds (Vibra cell™, Sonics&Materials Inc., Danbury, CT) and then 0.5 ml was used for traditional plate culturing methods. A spiral plating machine (Spiral System®, Microbiology International, Frederick, MD) was used to plate 50 μl inoculations on duplicate plates. Four mitis salivarius agar plates (Difco™ Mitis Salivarius Agar, Becton, Dickinson and Company, Sparks, MD) and four Gold’s agar (16) plates were individually used for culturing total streptococci and *S. mutans*, respectively for four ten-fold dilutions of each plaque sample. Following anaerobic incubation at 37°C for 48 hours, total streptococci and *S. mutans* were counted according
to the instructions for the spiral plater to enumerate the CFU/ml from each sample. Results were reported as the ratio of \( S. mutans \)/total streptococci expressed as a percent.

**Statistics**

Descriptive statistics assessed the means, medians, percentiles and variability of the basic samples by sample protocol (I or P) and derived variables (i.e., the mean of the 4 individually sampled teeth compared to the pooled sample, the use of the maximum value amongst the 4 teeth and/or the median value amongst the 4 teeth). Graphic plots were used to assess the homogeneity of the sample results and comparisons between the sampling methods in bivariate plots of paired measures. Test-retest reliability was assessed by comparing first and second samples of each method. Reproducibility was assessed by repeated measures ANOVA using SAS PROC MIXED to estimate and adjust for the intraclass correlation amongst the samples within the same patient taking into account the order of sampling. Statistical contrasts were used to analyze the level of difference between the “I” experimental group and the “P” experimental group as it relates to \( S. mutans \) per total streptococci counts. McNemar’s test was also used to determine the probability of detecting any \( S. mutans \) in the paired pooled versus individual sampling methods.

**Results**
A total of 35 subjects were recruited. The gender distribution was 54% male and 46% female. Race distribution was 11% Caucasian, 29% African American, 11% Hispanic, and 46% Asian. Fifty-four percent of the subjects had a self reported past history of caries. Results were similar between adults and children and were combined for simplicity.

The average percent of *S. mutans*/total streptococci was 1.64% (+/-4.43%) and 1.21% (+/-3.17%) from pooled and individual plaque samples, respectively. Overall, most of the subjects harbored less than 1% of *S. mutans* per total streptococci; however, some subjects harbored high *S. mutans* ratio to total streptococci (i.e., greater than 10% of *S. mutans*).

Comparison of individual samples using one value per person for percentage of *S. mutans* versus total streptococci was accomplished using a paired *t*-test both within the groups and by collection order (i.e., first versus second and individual versus pooled samples). The results revealed no significant difference (p>0.05) comparing the pooled sampling method versus individual sampling method within groups and/or orders, although individual sampling method showed less variation as it represents the average of more samples than the single pooled sample.

While the results did not identify clear difference indicative of a preference for the sampling method based on the levels of SM detected given the sample size available for this study, it was of interest to determine if one method (pooled sampling of 4 molars versus 4 individual samples) was more sensitive in detecting *S. mutans* within an individual. Twelve of the 35 subjects had no detectable *S. mutans* from any plaque
samples. Furthermore, 8 of the subjects had detectable *S. mutans* from both sampling methods (i.e., from 8 individual and 2 pooled samples). Therefore, 57% (20 of 35 totals) of the individuals resulted in the same conclusion as to the question of whether *S. mutans* was present in the plaque of the permanent first molars. However, differences were observed in detection of *S. mutans* colonization with the two methods of sampling among the other 15 subjects (43%). Table 3 shows that of these discrepancies in detection of *S. mutans*, four times more subjects were found to be colonized with *S. mutans* using the 8 individual samples compared to the two pooled samples (12 subjects colonized versus 3 colonized, respectively). These findings indicate individual sampling is more sensitive in detecting *S. mutans* than the pooled sampling method (p=0.02, McNemar’s test). Table 4 shows that even if only 4 samples are collected individually compared to 2 pooled samples, twice the number of subjects with *S. mutans* were found to be colonized with *S. mutans* (i.e., 10 versus 5, p>0.05). These results were consistent with the suggestion that individual sampling method is superior to pooled samples.

**Discussion**

In general, the study demonstrated two important results regarding the efficacy and sensitivity of the plaque sampling methods. First, there was no statistically significant difference between the two sampling methods in our study on the mean ratio of *Sm*/total streptococci. However, the individual sampling method does show slightly less variation in both age groups. Second, the individual sampling method offers more opportunities for detecting *S. mutans* than the pooled sampling method; however, the price paid for this
increased efficiency is substantially increased workload and related financial costs, in
terms, time, personnel, and recourses. This is an important consideration in a clinical
study, especially with large sample sizes. For population surveys, the less costly pooled
samples, while they may underestimate the true colonization rate, may be sufficient to
track a population or examine subgroups or trends. However, for prospective studies of
incidence or the timing of the onset of infection, the individual samples are likely
preferred if the resources available can support its implementation. The subject
population in this study was composed of multiracial population (11% Caucasian, 29%
African American, 11% Hispanic, and 46% Asian), it may not represent the majority of
the population in the community and may just reflect the word of mouth sampling.
However, the diversity also suggests it may be more widely representative.

Regarding these findings, the lack of a statistically significant difference in mean
levels between the two methods can be attributed to great variability in the collection
process, the oral environment, sample processing afterward and our limited sample size.
As mentioned in the Introduction, it is well-known that the oral cavity is a dynamic and
complicated environment, harboring over 500 bacterial species. Even though in some
ways plaque samples are not as complicated as saliva samples, they remain under the
same influences of this highly active environment. Additionally, different collection
surfaces of teeth serve as variables as well, regardless of which collection methods are
used. Although the protocol designed attempted to standardize the collecting procedures
and ensure that all the surfaces were sampled, there is no guarantee that plaque collected
from different surfaces were equally distributed (18) and that colonization occurs in an
expected regular or uniform pattern.
Another important factor that could contribute the observed sample variability is the sample processing procedure. In this study, we use the standard plate count (SPC) method, which has been the gold standard for processing oral samples since 1960s. However, this method contains many variations within the multiple procedures, such as different plating systems and dilutions as well as variability from plating to plating, subjectivity of counts by observer, and loss of viability of bacterial due to sonication. Also, the result from SPC was assumed that each colony on the plate represents one cell of the organism, which cannot be easily verified. These variations reduce the reliability of the bacterial count from SPC, even though previous research promoted this method as having greater than 90% of sensitivity for \textit{S. mutans} (19).

In this study, we attempted to identify the most sensitive and efficient means of sample collection by determining which method resulted in lower variation. Considering all possible variables involved and our limited sample size, it is not surprising to see the lack of statistical differences between the methods. SPC is a traditional method to quantify \textit{S. mutans} from oral samples. While this study demonstrated a number of shortcomings in the plaque sampling and that pooled samples may not be an accurate means to study the level of oral colonization, newer leading-edge technologies, such as real-time PCR may provide a better alternative to SPC. In the last 15 years, this technology has increased the accuracy and precision of PCR quantitation using precisely controlled conditions that allow monitoring of the propagation of DNA product by targeting specific DNA in microorganisms, and, in advance, quantifying the bacterial numbers (20). Increasingly, these technologies may be applied in the quantitation of oral microorganisms without compromising the accuracy and precision in the future.
As for detecting bacteria, we found individual sampling superior to pooled sampling as a detection method for *S. mutans*. When examining the discordant detection of MS, the presence of undetected MS from the pooled samples yielded four times more subjects that were identified having *S. mutans* using the individual sampling method (Table 3) than the pooled sample compared to the individual sampling. This finding implies that collecting plaque samples from each individual tooth offers more opportunities to detect *S. mutans* if the purpose is strictly finding any *S. mutans* in oral samples (if *S. mutans* is present) regardless of other requirements. Detection of *S. mutans* may be very important to provide an opportunity to characterize this potential pathogen (i.e., by genotyping) early in the disease process during longitudinal studies whereby later more *S. mutans* may be present. It will also provide an opportunity to compare these characteristics to assess stability, diversity and ultimately pathogenicity of the infecting organism. However, by increasing number of samples collected, there is an increased load on lab processing and financial budgets depending on the number of subjects involved as mentioned earlier.

Previous studies on quantitation of *S. mutans* have emphasized more the discovery of selective media to increase sensitivity of recovering *S. mutans* from oral samples. Nevertheless, there is lack of studies that have focused on the possible effect of different sampling methods in plaque samples. While our sample size was limited (35 subjects), our finding illustrates that *S. mutans* counts are influenced by different collection methods, offering new considerations for practitioners and researchers, to consider carefully the methods of sample collection depending upon their situations and purposes.
In conclusion, similar variability was observed with these two sampling methods in collecting plaque samples; however, individual sampling method was found to be superior detecting *S. mutans*. Although the individual sampling method was found to be superior in detecting *S. mutans* in plaque samples, the four times the workload of processing samples may be a factor to consider if a large-scale study is to be conducted. Therefore, a targeted approach to plaque sampling based on different clinical and research circumstances is needed. If subjects for the study are not difficult to recruit (i.e., large sample size possible) or a relatively high *S. mutans* colonization is expected, it might be preferable to use the pooled sampling method for each subject to accommodate the size and scope of the study in cost-effective manner (i.e., collecting 4 times the number of samples pooled as opposed to individual sampling of smaller numbers of subjects). On the other hand, if subjects of the study are difficult to recruit, such as patients with special conditions, or lower *S. mutans* counts are expected, it might be worthwhile to use the individual sampling method and collect more oral samples from each subject, gaining increased precision in the *S. mutans* counts.

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References


**Figure Legend:** Illustration of sample collection order in division of “I” and “P” group.

Each subject was randomly assigned to Group “I” or “P”. Group I had 10 samples collected in the order illustrated beginning with a plaque sample from tooth #3. Similarly, group P had 10 samples collected but began with a pooled sample of all four permanent first molars.
**Table 1: Demographic Data**

<table>
<thead>
<tr>
<th></th>
<th>Subjects</th>
<th>Percentage</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult</td>
<td>Children</td>
<td>Adult</td>
</tr>
<tr>
<td><strong>Mean Age</strong></td>
<td>31.8yo</td>
<td>8.8yo</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>11</td>
<td>47%</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>7</td>
<td>53%</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>2</td>
<td>2</td>
<td>12%</td>
</tr>
<tr>
<td>African American</td>
<td>3</td>
<td>7</td>
<td>18%</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0</td>
<td>4</td>
<td>0%</td>
</tr>
<tr>
<td>Asian</td>
<td>12</td>
<td>4</td>
<td>71%</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>1</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Caries History</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Past Caries History</td>
<td>10</td>
<td>9</td>
<td>59%</td>
</tr>
<tr>
<td>No Caries History</td>
<td>7</td>
<td>9</td>
<td>41%</td>
</tr>
</tbody>
</table>
### Table 2: Distribution of Sm/TS* Ratio

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean Percent Sm/TS (s.d.)</th>
<th>Pooled Sampling</th>
<th>Individual Sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1st set</td>
<td>2nd set</td>
</tr>
<tr>
<td>Adults</td>
<td>17</td>
<td></td>
<td>0.95(2.99)</td>
<td>0.8(2.31)</td>
</tr>
<tr>
<td>Children</td>
<td>18</td>
<td></td>
<td>3.24(10.96)</td>
<td>1.50(3.06)</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td></td>
<td>2.13(8.10)</td>
<td>1.16(2.71)</td>
</tr>
</tbody>
</table>

* Sm/TS: S. mutans divided by total streptococci x 100%
**Table 3**: Detection of *S. mutans* using eight individual samples versus two pooled samples

<table>
<thead>
<tr>
<th></th>
<th>Average of 8 Individual Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
</tr>
<tr>
<td><strong>Average of 2</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Pooled Samples</strong></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>8</td>
</tr>
<tr>
<td>Not Infected</td>
<td>12*</td>
</tr>
</tbody>
</table>

*p* = 0.02, significant difference, McNemar’s test
**Table 4:** Detection of *S. mutans* using four individual samples versus two pooled samples

<table>
<thead>
<tr>
<th>Average of 2 Pooled Samples</th>
<th>Average of 4 Individual Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
</tr>
<tr>
<td>Infected</td>
<td>6</td>
</tr>
<tr>
<td>Not Infected</td>
<td>10*</td>
</tr>
</tbody>
</table>

* p > 0.05, no significant difference, McNemar’s test
CONCLUSION

Dental caries is an infectious and worldwide prevalent disease. In the US, it is seven times more common than asthma. Although dental caries is preventable, there are few diagnostic tools to aid clinicians in accomplishing an accurate caries risk assessment in order to make a customized treatment plan and prevention program for each patient. Focusing on the infectious nature of dental caries is even less accurate. For example, taking one of the caries risk tests that is marketed, CRT bacteria, samples must be incubated for several days using the testing bar in an incubator and the colony counts are not accurate (21). Thus, since bacteria tests like this are not deemed practical, more time and expense is spent treating caries instead of focusing preventive measures based on caries risk. Developing a fast, easy and accurate caries risk assessment tool is necessary and would benefit not only the clinicians but also dental caries researchers.

The standard plate culture method has been the traditional way to quantifying *S. mutans* from oral samples. Although when developed in the 1960’s, it was reported that greater than 90% sensitivity to detect *S. mutans* could be expected, this method has come under criticism due to the high variability of counts, time and labor requirement, and the subjectivity of counts by observers. In an unpublished study in this laboratory, we investigated the potential of one of the leading-edge technologies, SYBR GREEN real-time PCR (i.e. quantitative PCR, Q-PCR), as an alternative quantitation method for *S. mutans*. We compared the bacterial counts from standard plate culture method and
quantitative PCR on ten replicates of the prototype *S. mutans*, strain UA 159, average OD$_{600}$ = 1.0 using flow cytometry to count cells. The result showed that bacterial counts from Q-PCR were significantly higher than from standard plate culture method (Appendix B, Table 1). However, the variation between ten replicates was higher in quantitative PCR than standard plate culture method (Appendix B, Table 2). This finding indicates that Q-PCR has the potential to replace the traditional plate culture method by its sensitivity if precision can be improved and verified, especially in consideration of its time efficiency. Future goals will be focused on decreasing the variation between process procedures also targeting multiple oral streptococci, such as *S. sobrinus* and *Streptococcus sanguinis*, which can be done in one experimental set-up (i.e., multiplex technology) from individual clinical oral samples.

Although it is important to have a clinical caries risk assessment that focuses on *S. mutans* colonization, it will not be accurate without a representative oral sample. Oral samples, including saliva and plaque, are the most common sample collected and used for a clinical caries assessment. Recently, research efforts aimed at salivary diagnostic technology development has been a priority. For example parotid saliva is used for oral cancer screening (22). However, standardization of methods to collect a representative sample remains a challenge. Representative plaque samples are more difficult and complex to collect than saliva samples due to multiple tooth surfaces and different tooth morphology. In our study, we found similar variability between the pooled sampling method and individual sampling method in proportion of *S. mutans* versus total streptococci regardless of ages of subjects and sample collection order. Nevertheless, the individual sampling method (i.e. collecting more samples) appeared to be more reliable in
detecting *S. mutans* from plaque samples. Although this finding indicates that individual sampling may be more sensitive in detecting *S. mutans* than the pooled sampling method (most likely because of the increased number of samples), it must also be realized that more labor and cost for sample processing are required. If we consider the cost/efficiency of sample processing, it may be worth collecting individual samples if subjects are rare and difficult to recruit, such as subjects with unusual conditions. On the other hand, since there is no significant difference in variability between individual and pooled sampling methods, if subjects are not difficult to recruit resulting in large sample sizes, it could be preferable to use a pooled sampling method due to the cost/efficiency matters. This notion is further supported by our study data (Appendix B, Table 3&4) when we compared just one individual sample and one pooled sample, regardless of the location or the collection order. These results imply that the same amount of the random error exists between individual and pooled samples. Please refer to the APPENDIX section for supported data.

In summary, this study was unable to find significant difference in pooled and individual sampling methods due to the high amount of variability. In addition, this study showed that more sampling (i.e. individual sampling) is more efficient in detecting *S. mutans* than one single pooled sampling. Future study would be preferable to test larger sample sizes and to use a more sensitive sample processing method, such as Q-PCR.
GENERAL LIST OF REFERENCES


APPENDIX A

IRB APPROVAL FORMS
Project Revision/Amendment Form

(Please type: In MS Word, highlight the shaded, underlined box and replace with your text; double-click checkboxes to check/uncheck.)

Link: Project Revision/Amendment Form

Federal regulations require IRB approval before implementing proposed changes.
Please complete this form and attach the changed research documents. Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the investigator’s brochure, questionnaires, surveys, advertisements, etc.)
Principal Investigator: Noel K. Childers, D.D.S., M.S., Ph.D. Date: May 11, 2006
Contact: Yvonne McLane Phone #: 4-1315 Fax #: 4-7073 E-mail: ymclane@uab.edu
Campus Address: SDB 304 - 0007
Study/Protocol Title: Liposomal Recombinant Vaccine and Caries Immunity: Aim 2
IRB Protocol #: X050126003

Current Status of Project: (check only one)
☐ Currently in Progress (# participants entered: ____ )
☐ Study has not yet begun (no participants entered)
☐ Closed to participant enrollment (remains active); # participants on therapy/intervention ____ ; # participants in long-term follow-up only ______

This submission changes the status of this study in the following manner:
(check all that apply)
☐ Protocol Revision
☒ Protocol Amendment
☐ Study Closed to participant entry
☐ Study Terminated
☐ Revised Consent Form
☐ Addendum (new) consent form
☐ Enrollment temporarily suspended by sponsor
☒ Other, (specify) Sponsor changed to UAB School of Dentistry. Department of Pediatric Dentistry

1. Briefly describe, and explain the reason for, the revision or amendment. Include a copy of supportive documents with changes highlighted. Please highlight changes/revisions/additions to the consent form, protocol, research questionnaire, etc.

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

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

4. Does the change affect the consent document?
If yes, briefly discuss the changes. Sponsor name change

Include the revised consent form with the changes highlighted. Will any participants need to be reconsented as a result of the changes? ☐ Yes ☒ No
If yes, when will participants be reconsented?

Signature of Principal Investigator: ______________ Date: 5/12/06

Revised 04-07-04
Federal regulations require IRB approval before implementing proposed changes. Please complete this form and attach the changed research documents. Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.)

Principal Investigator: Noel K. Childers Date: July 7, 2006
Contact: Yvonne McClelPhone #: 4-1315 Fax #: 4-7013 E-mail: ymcclel@uah.edu
Campus Address: SOC 304 - 0007
Study/Protocol Title: Liposomal Recombinant Vaccine and Caries Immunity: Aim 2
IRB Protocol #: X030126003

Current Status of Project: (check only one)

- [ ] Currently in Progress (# participants entered: 4)
- [ ] Study has not yet begun (no participants entered)
- [ ] Closed to participant enrollment (remains active); # participants on therapy/intervention 5; # participants in long-term follow-up only

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

- [ ] Protocol Revision
- [ ] Protocol Amendment
- [ ] Study Closed to participant entry
- [ ] Study Terminated
- [ ] Revised Consent Form
- [ ] Addendum (new) consent form
- [ ] Enrollment temporarily suspended by sponsor
- [X] Other, (specify) Change Oral Health Assessment Form

1. Briefly describe, and explain the reason for, the revision or amendment. Include a copy of supportive documents with changes highlighted. Please highlight changes/revisions/additions to the consent form, protocol, research questionnaire, etc.

Relative to the Project Revision that was approved June 21, 2006, the original questionnaire which was directed toward children needs to be revised for the adults that will participate in this study._____

2. Does this revision/amendment revise or add a genetic or storage of samples component?

- [ ] Yes
- [X] No

If yes, please see the Guidebook to assist you in revising or preparing your submission documents or call the IRB office at 4-3789.

3. Does the change affect subject participation (e.g. procedures, risks, costs, etc.)?

- [ ] Yes
- [X] No

If yes, briefly discuss the changes._____

4. Does the change affect the consent document?

- [ ] Yes
- [X] No

If yes, briefly discuss the changes._____

Include the revised consent form with the changes highlighted.

Will any participants need to be reconsented as a result of the changes?

- [ ] Yes
- [X] No

If yes, when will participants be reconsented?_____

Signature of Principal Investigator: Noel K. Childers Date: 7/7/06

Revised 04-07-04
Project Revision/Amendment Form

(PLEASE TYPE: In MS Word, highlight the shaded, underlined box and replace with your text; double-click on check/uncheck box.)

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

Today's Date: July 31, 2008

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

2. Protocol Identification
Protocol Title: Liposomal Recombinant Vaccine and Caries Immunity: Aim 2
IRB Protocol Number: 0050126003

Current Status of Project (check only one):
☐ Currently in Progress (Number of participants entered: _____)
☐ 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: ______
☐ Closed to participant enrollment (data analysis 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
☐ Study Closure
☐ Other, (specify)
☐ Revised Consent Form
☐ Addendum (new) consent form
☐ Enrollment temporarily suspended by sponsor
☐ Change in protocol personnel

3. Reason for change
Briefly describe, and explain the reason for, the change. If normal, healthy controls are included, describe in detail how this change will affect those participants.
Include a copy of the protocol and any other documents affected by this change (e.g., consent form, questionnaire) with all the changes highlighted. Please add Dr. Kei-Ling (Christine) Hu to this protocol as a co-investigator. Dr. Hu is up to date on her IRB Human Subjects training.

4. Does this change revise or add a genetic or storage of samples component?
☐ Yes ☐ No
If yes, please see the Guidebook to assist you in revising or preparing your submission, or call the IRB office at 934-3789.

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

6. Does the change affect the consent document(s)?
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
If yes, when will participants be reconsented?

Signature of Principal Investigator

Date 7/31/08

Amendment 7312008.doc
APPENDIX B

EXTRA SUPPORTED DATA
Table 1: Quantitation data of each replicate of UA159 from SPC and Q-PCR

<table>
<thead>
<tr>
<th>Replicates</th>
<th>OD600</th>
<th>SPC* (CFU/ml)</th>
<th>Q-PCR (Org/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.01</td>
<td>4.28E+08</td>
<td>9.56E+08</td>
</tr>
<tr>
<td>2</td>
<td>1.02</td>
<td>4.61E+08</td>
<td>7.56E+08</td>
</tr>
<tr>
<td>3</td>
<td>0.99</td>
<td>4.60E+08</td>
<td>6.83E+08</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
<td>4.22E+08</td>
<td>1.24E+09</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>4.27E+08</td>
<td>9.78E+08</td>
</tr>
<tr>
<td>6</td>
<td>1.01</td>
<td>3.83E+08</td>
<td>1.45E+09</td>
</tr>
<tr>
<td>7</td>
<td>1.01</td>
<td>3.52E+08</td>
<td>1.26E+09</td>
</tr>
<tr>
<td>8</td>
<td>1.00</td>
<td>3.16E+08</td>
<td>1.09E+09</td>
</tr>
<tr>
<td>9</td>
<td>1.00</td>
<td>3.98E+08</td>
<td>1.38E+09</td>
</tr>
<tr>
<td>10</td>
<td>1.01</td>
<td>4.30E+08</td>
<td>8.21E+08</td>
</tr>
</tbody>
</table>

Average 1.01 4.08E+08 1.06E+09

*SPC represents for Standard plate count
Table 2: Statistical analysis of quantitation data of ten replicates of UA159 from SPC and Q-PCR

<table>
<thead>
<tr>
<th>Measures</th>
<th>SPC</th>
<th>Q-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.08E+08</td>
<td>1.06E+09</td>
</tr>
<tr>
<td>STDEV</td>
<td>4.60E+07</td>
<td>2.39E+08</td>
</tr>
<tr>
<td>Coefficient of variation (%)</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td><em>P value</em></td>
<td>1.09E-04</td>
<td></td>
</tr>
</tbody>
</table>

* *p*<0.05, significant difference, t-test.
Table 3: Sampling effect comparison between individual and pooled samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>+ +</th>
<th>+ -</th>
<th>- +</th>
<th>- -</th>
<th>Agreement (%)</th>
<th>Disagreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual #3</td>
<td>11</td>
<td>6</td>
<td>3</td>
<td>15</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>#14</td>
<td>13</td>
<td>3</td>
<td>4</td>
<td>15</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>#19</td>
<td>12</td>
<td>2</td>
<td>3</td>
<td>18</td>
<td>86</td>
<td>14</td>
</tr>
<tr>
<td>#30</td>
<td>13</td>
<td>5</td>
<td>4</td>
<td>13</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>Pooled #Pool</td>
<td>17</td>
<td>4</td>
<td>3</td>
<td>11</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

+ +: 1<sup>st</sup> and 2<sup>nd</sup> samples both detected <i>S. mutans</i>
+ -: 1<sup>st</sup> sample detected <i>S. mutans</i>, 2<sup>nd</sup> sample did not detect <i>S. mutans</i>
- +: 2<sup>nd</sup> sample detected <i>S. mutans</i>, 1<sup>st</sup> sample did not detect <i>S. mutans</i>
- -: 1<sup>st</sup> and 2<sup>nd</sup> samples both did not detect <i>S. mutans</i>
Table 4: One sample comparison of *S. mutans* detection between individual and pooled samples

<table>
<thead>
<tr>
<th>Sample Comparison</th>
<th>+ +</th>
<th>+ -</th>
<th>- +</th>
<th>- -</th>
<th>Agreement (%)</th>
<th>Disagreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3 - #P</td>
<td>13</td>
<td>4</td>
<td>8</td>
<td>10</td>
<td>66</td>
<td>34</td>
</tr>
<tr>
<td>#14 - #P</td>
<td>13</td>
<td>3</td>
<td>8</td>
<td>11</td>
<td>69</td>
<td>31</td>
</tr>
<tr>
<td>#19 - #P</td>
<td>11</td>
<td>3</td>
<td>10</td>
<td>11</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>#30 - #P</td>
<td>14</td>
<td>4</td>
<td>6</td>
<td>11</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>Second Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3 - #P</td>
<td>13</td>
<td>0</td>
<td>7</td>
<td>15</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>#14 - #P</td>
<td>14</td>
<td>3</td>
<td>6</td>
<td>12</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>#19 - #P</td>
<td>13</td>
<td>2</td>
<td>7</td>
<td>13</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>#30 - #P</td>
<td>15</td>
<td>2</td>
<td>5</td>
<td>13</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

+ +: Individual (#3, 14, 19, 30) and pooled samples both detected *S. mutans*

+ -: Individual sample detected *S. mutans*, pooled sample did not detect *S. mutans*

- +: Pooled sample detected *S. mutans*, individual sample did not detect *S. mutans*

- -: Individual and pooled samples both did not detect *S. mutans*