Variability of Two Plaque Sampling Methods in Quantitation of *Streptococcus mutans*

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Key Words

Caries • Caries risk assessment • Mutans streptococci • Plaque • Sampling method • *Streptococcus mutans*

Abstract

The aim of this study was to determine the reproducibility of individual versus pooled plaque sampling of permanent first molars (PFM) to quantitate *Streptococcus mutans* (SM)/total streptococci (TS). Ten individual and pooled plaque samples were collected from 35 subjects, randomly assigned to individual-first or pooled-first group. Plaque samples were processed and quantified for SM and TS. SM/TS ratio was used to determine the reproducibility within two group samples. Mean percentage of SM/TS in both methods were not significantly different. However, within subject detection of SM was found to be significantly more sensitive for individual sampling method. Despite the lack of a difference between both methods for SM/TS quantitation, the difference in SM detection suggests that individual sampling is more sensitive.

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Dental caries is the most prevalent infectious disease in humans. The latest data from the US national survey indicates that while dental caries is decreasing overall,

young children (2–5 years old) are experiencing increased prevalence [Dye et al., 2007]. Among hundreds of bacterial species in the human oral cavity, overwhelming data supports colonization with mutans streptococci (MS) as having a major etiological role in dental caries initiation and development [Loesche, 1986]. MS, as it relates to the pathogenesis of dental caries in humans, are composed of *Streptococcus mutans* and *Streptococcus sobrinus* [Morrison et al., 1998; Tanzer et al., 1984]. Since *S. mutans* is most commonly isolated and associated with human samples, this species is the focus of most research [Loesche, 1986]. Many studies have identified the relationship between the activity of dental caries, the quantity of MS, and the timing of initial infection in the oral cavity [Loesche, 1986; Tanzer et al., 2001]. 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 common for clinicians 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 secretion rate, infusion of and duration of saliva contact as it interacts with biofilm on enamel surfaces lead to variability and lack of reproducibility. Dental plaque samples, on the other hand, are direct samples of MS containing substances, yet are relatively complicated

Fig. 1. Illustration of sample collection order in division of 'I' and 'P' groups. 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 No. 3. Similarly, group P had 10 samples collected but began with a pooled sample of all 4 permanent first molars.

to collect, due to variability in efficiency of amount collected and complexity in the number of teeth and the surfaces available for collection [Dasanayake et al., 1995]. In this regard, the nature of plaque, which is a dynamic accumulation of organisms on tooth surfaces, presents a problem 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 and processing. Therefore, due to the nature of plaque collection, if methods are not optimally standardized, studies that use this method can yield conflicting information. Given the possibility of variation that may be seen from plaque sampling methods, it is reasonable to determine the superiority of one collection method over the other as it pertains to obtaining consistent and reproducible detection of MS.

The purpose of this study was to compare two different plaque collection methods (i.e., individually sampled index teeth versus a pooled single sample collected from a number of index teeth) for quantifying *S. mutans* from permanent first molar teeth (PFM). We used a standard plate counting method to process plaque samples to evaluate the collection reproducibility of two methods, pooled and individual sampling from 4 PFM. The goal was to determine which method was most representative and reproducible for assessing plaque samples in a population to aid assessment of caries risk associated with *S. mutans* colonization.

Subjects and Methods

Experiment Design

Seventeen adults (age 17–50 years) and 18 children (age 6–12 years) were recruited, and consented for collection of plaque. Inclusion criteria stipulated that subjects be healthy, not take antibiotics within 2 weeks prior to the sample collection and with all four PFM fully erupted in the oral cavity. Dental exclusion criteria were the presence of obvious dental caries, periodontal disease, or other oral disease. A visual dental examination was completed to determine eligibility, and then plaque samples were collected using the IRB-approved protocol described below.

Plaque Collection Methods and Processing

Subjects were randomly assigned to either the 'I' group (i.e., 4 individual PFM tooth samples collected first, then a pooled sample was collected from all 4 PFM) or the 'P' group (pooled samples from the 4 PFM teeth collected first, then individual samples were collected). Ten plaque samples (i.e., 2 sets of individual and 2 pooled samples) were collected from all (buccal/mesial/distal/lingual/occlusal) surfaces of individual PFM or pooled (all 4) PFM molars using a sterile toothpick and transferred to sterile saline as illustrated in figure 1.

Plaque samples were sonicated for 20 s (Vibra cell™, Sonics & Materials Inc., Danbury, Conn., USA) and then 0.5 ml was used for traditional plate culturing methods. A spiral plater (Spiral Systems Inc., Cincinnati, Ohio, USA) was used to plate 50 µl on duplicate plates. Four mitis salivarius agar plates (Difco™ Mitis Salivarius Agar, Becton, Dickinson & Co., Sparks, Md., USA) and four Gold's agar plates [Gold et al., 1973] were individually used for culturing total streptococci and *S. mutans*, respectively for four tenfold dilutions of each plaque sample. Following anaerobic incubation at 37°C for 48 h, total streptococci and *S. mutans* were counted according to the instructions for the spiral plater to enumerate the colony-forming units per milliliter from each sample.

Table 1. Distribution of SM/TS ratio

Group	n	Mean percent <i>S. mutans</i> divided by total streptococci $\times$ 100% (SD)					
		pooled sampling			individual sampling		
		1st set	2nd set	average	1st set	2nd set	average
Adults	17	0.95 (2.99)	0.80 (2.31)	0.88 (2.63)	0.49 (0.95)	2.53 (8.22)	1.51 (4.40)
Children	18	3.24 (10.96)	1.50 (3.06)	2.37 (5.62)	0.78 (1.19)	1.07 (1.78)	0.92 (1.29)
Total	35	2.13 (8.10)	1.16 (2.71)	1.64 (4.43)	0.64 (1.08)	1.78 (5.82)	1.21 (3.17)

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 sample-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 $1.64 \pm 4.43\%$ and $1.21 \pm 3.17\%$ from pooled and individual plaque samples, respectively (table 1). These results were not significantly different ($p > 0.05$) when comparing the pooled versus individual sampling methods within groups and/or orders, although individual sampling resulted in less variation than pooled samples.

While the results did not identify a clear difference indicative of a preference for the sampling method based on the levels of *S. mutans* detected, it was of interest to determine if one method (pooled versus individual samples) was more sensitive in detecting *S. mutans* within an individual. Total agreement with sampling methods was observed with 12 of the 35 subjects that had no detectable *S. mutans* from any plaque samples, while 8 subjects had detectable *S. mutans* from both methods (i.e., from 8 individual and 2 pooled samples). Therefore, 57% (20 of 35 totals) of the individuals resulted in agreement with the question of whether *S. mutans* was present in the plaque of the permanent first molars. However, differences (disagreement) were observed in detection of *S. mutans* colonization with the two methods of sampling among the other 15 subjects (43%). There was no observable association of *S. mutans*/total streptococci ratio and agreement in detection of *S. mutans* with the two methods of sampling. Four times more subjects were found to be colonized with *S. mutans* using the 8 individual samples compared to the 2 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). Even if only 4 samples were collected individually compared to 2 pooled samples, twice the number of subjects with *S. mutans* was found to be colonized with *S. mutans* (i.e., 10 vs. 5, $p > 0.05$).

Discussion

This 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 for the mean ratio of *S. mutans*/total streptococci. However, the

individual sampling method resulted in slightly less variation. 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 a substantially increased workload and related financial costs, in terms of time, personnel, and resources. 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, individual samples are preferred if the resources available can support its implementation.

Regarding these findings, the lack of a statistically significant difference in mean levels between the two methods could be attributed to great variability in the collection process, the oral environment, sample processing afterwards and our limited sample size. It is well known that the oral cavity is a dynamic and complicated environment, harboring over 500 bacterial species. Plaque samples remain under the influence 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 was equally distributed [Babaahmady et al., 1997] and that colonization occurs in an expected regular or uniform pattern.

Another important factor that could contribute to 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 may contain many variations within the multiple procedures, such as different plating systems and dilutions as well as variability from plate to plate, subjectivity of counts by observer, and loss of viability of bacteria due to sonication. Also, the SPC method assumes 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 *S. mutans* [Tanzer and Clive, 1986]. While this study may demonstrate a number of shortcomings in the plaque sampling and suggest 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 [Heid et al., 1996]. 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 4 times more subjects that were identified having *S. mutans* using the individual sampling method than the pooled 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. However, by increasing the 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 [Emilson and Bratthall, 1976; Gold et al., 1973]. Nevertheless, there are few studies that have focused on the possible effect of different sampling methods in plaque samples [Dasanayake et al., 1995; Wennerholm et al., 1995]. 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 individual versus pooled sampling methods in collecting plaque samples; however, individual sampling method was found to be superior in detecting *S. mutans*. Although the individual sampling method was found to be superior, if a large-scale study is to be conducted, the costs

of 4 times the workload of processing samples may be a factor to consider. Therefore, a targeted approach to plaque sampling based on different clinical and research circumstances is needed.

Acknowledgments

The authors wish to thank Drs. Tabitha Jarman and Bibi Rahima for their help and support. This study was supported by the Department of Pediatric Dentistry, University of Alabama at Birmingham, School of Dentistry, and a grant from the National Institute of Dental and Craniofacial Research (No. DE016684).

References

- Babaahmady KG, Marsh PD, Challacombe SJ, Newman HN: Variations in the predominant cultivable microflora of dental plaque at defined subsites on approximal tooth surfaces in children. *Arch Oral Biol* 1997;42:101–111.
- Dasanayake AP, Caufield PW, Cutter GR, Roseman JM, Kohler B: Differences in the detection and enumeration of mutans streptococci due to differences in methods. *Arch Oral Biol* 1995;40:345–351.
- Dye BA, Tan S, Smith V, et al: Trends in oral health status: United States, 1988–1994 and 1999–2004. *Vital Health Stat* 2007;11:1–92.
- Emilson CG, Bratthall D: Growth of *Streptococcus mutans* on various selective media. *J Clin Microbiol* 1976;4:95–98.
- Gold OG, Jordan HV, Van Houte J: A selective medium for *Streptococcus mutans*. *Arch Oral Biol* 1973;18:1357–1364.
- Heid CA, Stevens J, Livak KJ, Williams PM: Real time quantitative PCR. *Genome Res* 1996;6:986–994.
- Loesche WJ: Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 1986;50:353–380.
- Morrison TB, Weis JJ, Wittwer CT: Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* 1998;24:954–958, 960, 962.
- Tanzer JM, Livingston J, Thompson AM: The microbiology of primary dental caries in humans. *J Dent Educ* 2001;65:1028–1037.
- Tanzer JM, Borjesson AC, Laskowski L, Kurasz AB, Testa M: Glucose-sucrose-potassium tellurite-bacitracin agar, an alternative to mitis salivarius-bacitracin agar for enumeration of *Streptococcus mutans*. *J Clin Microbiol* 1984;20:653–659.
- Tanzer JM, Clive J: Quantitative considerations in microbiological evaluations for caries: risks for type II errors resulting from use of MSB agar. *Oral Microbiol Immunol* 1986;1:28–30.
- Wennerholm K, Lindquist B, Emilson CG: The toothpick method in relation to other plaque sampling techniques for evaluating mutans streptococci. *Eur J Oral Sci* 1995;103:36–41.