Comparison of Evaluating The Ratio of Cariogenic Bacteria in Plaque and Saliva Samples

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Abstract

Background: This study aimed to conduct a comparative analysis for evaluating the ratio of cariogenic bacteria in plaque and saliva samples as a preliminary study for developing a simple culture kit for caries-risk assessment. Materials and Methods: Stimulated saliva and plaque samples using sterile toothbrushes were obtained from adult volunteers. The ratio of Streptococcus mutans (*S. mutans*, Sm) to total streptococci (TS) (Sm/TS ratio) was determined by counting the number of colonies by the culture method. Results: The number of *S. mutans*, Sm/TS ratio (%), and plaque Sm%/saliva Sm% were significantly higher in plaque than in saliva. Moreover, there was a positive correlation between the number of *S. mutans* in plaque and saliva as well as between the Sm/TS ratios (%) in plaque and saliva. Conclusion: Although distinct positive correlations were observed between *S. mutans* levels in plaque and saliva, some subjects showed dissimilarities. The use of plaque samples, in which bacteria are detected at higher levels, is preferred because *S. mutans* levels were higher in plaque than in saliva, both in terms of the number of *S. mutans* and Sm/TS ratio. Moreover, the plaque to saliva ratios (plaque/saliva) in all subjects demonstrated better outcomes for the Sm/TS ratio than for the number of *S. mutans*, suggesting that plaque samples are more suitable than saliva samples for caries-risk assessment.

Key Words: Ratio of cariogenic bacteria, Streptococcus mutans, Caries-risk assessment, Epidemiology, Public health, Plaque, Saliva

Introduction

Streptococcus mutans (*S. mutans*) is closely associated with the initiation and progression of dental caries and is generally considered the principal cariogenic microorganism in humans [1,2]. Many researches on the frequency and distribution of *S. mutans* and its correlation with dental caries have been reported [3-9]. Previous large-scale studies have linked *S. mutans* to crown caries in children and adolescents [10,11] as well as to root caries in the elderly [12].

Plaque has the highest cariogenic bacterial content and is thus an ideal sample for assessing caries activity; however, it is difficult to quantify plaque samples [2,13]. Therefore, paraffin- or chewing gum-stimulated saliva is more commonly used to estimate the risk of dental caries [14–16]. Among the plaque sampling methods in use, suspensions of plaque samples collected by brushing (brushing plaque sample) can be used for quantitation and simple analysis, suggesting the clinical utility of these samples [17]. Cariogenic bacteria are frequently assessed according to the number of bacteria. However, few studies have investigated the utility of the ratio of mutans streptococci (MS) to total streptococci (TS) (MS/TS ratio) or the ratio of *S. mutans* to TS (Sm/TS ratio) as alternative methods for evaluating cariogenic bacteria [17,18]. Based on this background, we reported the usefulness of analysis of cariogenic bacteria to detect subjects with the severity of dental caries according to the Sm/TS ratio using dental plaque samples [19,20].

This report is a preliminary study aimed at developing a simple culture kit for assessing the caries risk based on the ratio of cariogenic bacteria in plaque samples. However, comparative analyses of plaque and saliva (which is frequently used as a sample) should be conducted to determine the ratio of cariogenic bacteria in plaque as data for examining cariogenic activities or associated caries risk. To date, no study has reported the correlation and distribution of S. mutans and conducted an analysis of the ratio of cariogenic bacteria in humans using saliva and plaque samples. In the present study, a comparative analysis for evaluating the ratio of cariogenic bacteria in saliva and plaque samples was conducted as a preliminary study aimed at developing a simple culture kit for caries-risk assessment.

Materials and Methods

Subjects and sample preparation

A cross-sectional study of an adult population sample was conducted at Nihon University School of Dentistry in Matsudo, Japan. In total, 134 adult volunteers aged 21–35 years in good physical condition and oral health was enrolled as experimental subjects. We collected samples at our university. Subjects with any systemic disease, those using medications affecting salivary secretion, and those taking antibiotics were excluded from the study. The selected subjects were instructed not to eat, drink, use a mouth wash, or smoke 3 h prior to their appointment. The aim of this study was informed in advance to subjects, and informed consent was obtained from each subject. This study was conducted with the approval of the Ethics Committee of the Nihon University School of Dentistry, Matsudo, Japan.

Oral samples of stimulated saliva and brushing plaque were successively collected from each subject using the following methods. Saliva stimulated by chewing paraffin gum was

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secreted over a period of 5 min while being collected in a sterile bottle, chilled in ice, and then used as a stimulated saliva sample. After the subjects rinsed their mouths with drinking water, a large amount of plaque was removed from their teeth by vigorous brushing for 1 min using a sterile toothbrush, collected into a sterile bottle for 30 s via a mouth rinse with 5 ml phosphate-buffered saline, and then used as a brushing plaque sample [17,19,20].

Bacterial analysis

Mitis Salivarius agar (Difco, Detroit, MI, USA), containing 20% sucrose and 0.25 U bacitracin, and 1% tellurite, supplemented with 20 g/L yeast extract, 10 g/L colistin, 10 g/L nalidixic acid, and 4 g/L gramicidin, was used as a selective medium for TS and MS, respectively [21]. Within 3 h of sampling, clinically isolated samples were disrupted by sonication (50 W, 20 s) using an ultrasonic apparatus (5202 Type, Otake Works, Tokyo, Japan), serially diluted with chilled brain heart infusion broth, and inoculated on selective media using a spiral plating system (Model-D, Gunze Sangyo, Inc., Tokyo, Japan). The number of TS and *S. mutans* colonies on plates were counted following anaerobic incubation for 48 hours. *S. mutans* could be visually distinguished according to the colony morphology on the agar plates. The ratio of *S. mutans* to TS was determined by counting the colonies and was designated as the Sm/TS ratio [17-20].

Statistical analysis

Descriptive statistics and statistical analyses were performed using IBM SPSS Statistics software, version 22.0 (SPSS IBM Corp, Chicago, IL, USA). The Wilcoxon signed-rank test was used to compare the mean values between the two groups, and the Spearman correlation coefficient was used for correlation analysis. Data were presented as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

Results

The mean number of *S. mutans* (10^5 colony-forming units (CFU)/mL) (mean ± SD) in plaque and saliva samples were 24.33 ± 69.67 and 8.01 ± 23.82, respectively. The Sm/TS ratios (%) in plaque and saliva samples were 2.97 ± 5.91 and 0.86 ± 3.14, respectively. *Figure 1* shows the distribution in the Sm/TS ratios (%) in plaque and saliva samples.The distribution of the Sm/TS ratios (%) in plaque and saliva samples were 0%–0.25% (44,92), 0.25%–0.5% (9,13), 0.5%–0.75% (15,2), 0.75%–1.0% (10,7), 1.0%–1.25% (1,5), 1.25%–1.5% (8,0), 1.5%–1.75% (2,2), 1.75%–2.0% (5,2), and 2.0% (40,11), respectively. The distribution of levels the Sm/TS ratios (%) was higher in plaque than in saliva.

The mean number of *S. mutans* (10^5 CFU/mL) (mean ± SD) was significantly greater in plaque than in saliva. The Sm/TS ratios (%) were significantly greater in plaque than in saliva ($p < 0.001$; Wilcoxon signed-rank test, *Table 1*).

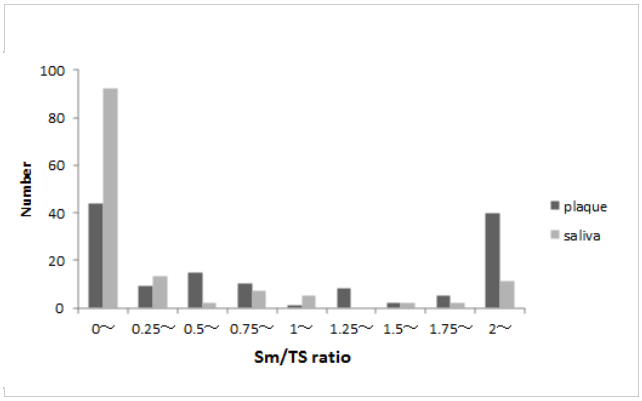

Figure 1. *The distribution of the Sm/TS ratios (%) in plaque and saliva samples.*

Table 1. *The mean number of S. mutans and the Sm/TS ratios (%) in plaque and saliva.*

	plaque	saliva
Sm/TS raito(%) (Mean ± SD)	2.97 ± 5.91	0.86 ± 3.14
	***	
The number of *S.mutans* 10^5 (CFU)/mL (Mean ± SD)	24.33 ± 69.67	8.01 ± 23.82
	***	

***: p< 0.001

Table 2. *Correlation between the number of S. mutans in plaque and saliva samples as well as between the Sm/TS ratios (%) in plaque and saliva samples.*

	correlation coefficient	
Sm/TS raito(%)	r=0.67	***
The number of S.mutans 10^5 (CFU)/ml	r=0.71	***

***: p< 0.001

Table 3. *The Sm/TS ratios in plaque and saliva samples (plaque Sm%/saliva Sm%) and the ratio of the number of S. mutans in plaque and saliva (plaque Sm/saliva Sm).*

	Mean ± SD	
Sm% in plaque /Sm% in saliva	20.56 ± 67.49	***
S. mutans in plaque / *S. mutans* in saliva	9.06 ± 15.95	

***: p< 0.001

There was a positive correlation between the number of *S. mutans* (10^5 CFU/mL) in plaque and saliva samples (r = 0.71; p < 0.001) as well as between the Sm/TS ratios (%) in plaque and saliva samples (r = 0.67; p < 0.001; Spearman correlation test, *Table 2*).

The Sm/TS ratios in plaque and saliva samples (plaque Sm %/salivaSm %) and the ratio of the number of *S. mutans* in plaque and saliva (plaque Sm/saliva Sm) were 20.56 ± 67.49 and 9.06 ± 15.95, respectively, demonstrating a significant difference between the two groups (p < 0.001; Wilcoxon signed-rank test, *Table 3*).

Discussion

The oral cavity contains many microbial species, making oral flora one of the most complex bacterial communities in humans [22,23]. Saliva promotes bacterial transmission and acts as a reservoir for colonization [24], because many bacteria (including anaerobes) can survive in saliva and utilize its components for growth [25,26]. Saliva allows dental plaque to flourish and detaches layers of plaque-releasing bacteria [27-29]. Thus, the salivary levels of certain bacteria may reflect the presence of these microorganisms in plaque [30,31]. In this context, a significant correlation has been reported between the salivary levels of MS and the proportions of these bacteria in the plaque flora [14,32]. Accordingly, the salivary levels of cariogenic bacterial species have been investigated as potential markers for caries-risk assessment [14,33].

Plaque containing MS are preferred for use as samples for caries-risk assessment [2,13]. However, it is challenging to quantitatively measure plaque samples, and thus, saliva is frequently stimulated by mastication in clinical practice [14–16], because saliva reflects the bacterial flora present in plaque. In the present study, we used a brushing plaque sample that was quantitatively measured [17,19,20]. Although we observed high correlations between the bacterial levels in plaque and saliva samples (approximately r = 0.7), some subjects showed dissimilarities. The use of plaque samples is considered to be more effective for increasing the accuracy of caries-risk assessment.

Simple and commercially available culture kits such as Dentocult SM™ utilize saliva, and they have been shown in clinical and epidemiological studies to detect MS at the chairside without the need for expensive equipment [2,14-16]. In the present study, we used a similar culture medium for comparative analysis with simple commercial kits, enabling us to gather data for developing a simple culture kit using the ratio of cariogenic bacteria in plaque for caries-risk assessment.

In this study, the number of *S. mutans* detected in saliva was 8.01 (10^5 CFU/mL) with an average Sm/TS ratio of 0.86%, whereas the number of *S. mutans* detected in dental plaque was 24.33 (10^5 CFU/mL) with an average Sm/TS ratio of 2.97%. The detection levels using the Sm/TS ratio and number of *S. mutans* in dental plaque were 3.5- and 3.0-fold higher, respectively, than those for *S. mutans* in saliva. Furthermore, the mean comparison ratios between cariogenic bacteria in plaque and saliva samples of subjects in terms of plaque Sm%/saliva Sm% and plaque Sm/saliva Sm were 20.6 and 9.1, respectively, showing a better outcome for Sm/TS ratio. This strongly suggests that the occurrence of *S. mutans* significantly varies among subjects and that plaque samples are more suitable than saliva samples for caries-risk assessment. We will conduct further research with a larger number of subjects, to establish the evaluation criteria for the ratio of cariogenic bacteria in saliva and plaque, and we will develop a simple culture kit using plaque samples for caries-risk assessment.

Conclusion

In the current study, we examined the distribution and correlation of the number of *S. mutans* and Sm/TS ratio in plaque and saliva. Although distinct positive correlations between the *S. mutans* levels in plaque and saliva were observed, some subjects showed dissimilarities. The use of plaque samples, in which bacteria could be detected at higher levels, was preferred.

The S. mutans levels were higher in plaque than in saliva in terms of the number of *S. mutans* and the Sm/TS ratio. Moreover, the plaque to saliva ratios in all subjects (plaque/saliva) demonstrated better outcomes for Sm/TS ratio than for the number of *S. mutans*, suggesting that plaque samples are more suitable than saliva samples for caries-risk assessment.

Conflict of interest

There are no conflicts of interest to declare.

References

1. Hamada S, Slade HD. Biology, immunology, and cariogenicity of Streptococcus mutans. *Archive of Microbiological Reviews.* 1980; **44**: 331-384.

2. Axelsson P (2000) Diagnosis and risk prediction of dental caries. Quintessence, Illinois, United States.

3. Singh N, Chawla HS, Tewari A, Sachdev V. Correlation of severity of streptococcus mutans in the saliva of school children of Chandigarh with dental caries. *Baba Farid University Dental Journal.* 2010; **1**: 5-8.

4. Okada M, Taniguchi Y, Hayashi F, Doi T, Suzuki J, et al. Late established mutans streptococci in children over 3 years old. *International journal of Dentistry.* 2010; **10**: 732468.

5. Edelstein BL, Ureles SD, Smaldone A. Very high salivary Streptococcus mutans predicts caries progression in young children. *Pediatric Dentistry.* 2016; **38**: 325-330.

6. Latifi-Xhemajli B, Véronneau J, Begzati A, Bytyci A, Kutllovci T, et al. Association between salivary level of infection with Streptococcus mutans/Lactobacilli and caries-risk factors in mothers. *European Journal of Paediatric Dentistry.* 2016; **17**: 70-74.

7. Drake D, Dawson D, Kramer K, Schumacher A, Warren J, et al. Experiences with the Streptococcus Mutans in Lakota Sioux (SMILeS) Study: Risk factors for caries in American Indian children 0-3 years. *Journal of Health Disparities Research and Practice.* 2016; **8**: 123-132.

8. Karjalainen S, Tolvanen M, Pienihäkkinen K, Söderling E, Lagström H, et al. High sucrose intake at 3 years of age is associated with increased salivary counts of mutans streptococci and lactobacilli, and with increased caries rate from 3 to 16 years of age. *Caries Research.* 2015; **49**: 125-132.

9. Abbate GM, Borghi D, Passi A, Levrini L. Correlation between unstimulated salivary flow, pH and Streptococcus mutans, analysed with real time PCR, in caries-free and caries-active children. *European Journal of Paediatric Dentistry.* 2014; **15**: 51-54.

10. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. *Journal of Clinical Microbiology.* 2008; **46**: 1407-1417.

11. Corby PM, Lyons-Weiler J, Bretz WA, Hart TC, Aas JA, et al. Microbial risk indicators of early childhood caries. *Journal of Clinical Microbiology.* 2005; **43**: 5753-5759.

12. Preza D, Olsen I, Aas JA, Willumsen T, Grinde B, et al. Bacterial profiles of root caries in elderly patients. *Journal of Clinical Microbiology.* 2008; **46**: 2015-2021.

13. Li Y, Tanner A. Effect of antimicrobial intervention on oral microbiota associated with early childhood caries. Pediatric Dentistry. 2015; 37: 226-244.

14. Guo L, Shi W. Salivary biomarkers for caries risk assessment. *Journal of California Dental Association.* 2013; **41**: 107-118.

15. Parisotto TM, Steiner-Oliveira C, Silva CM, Rodrigues LK, Nobre-dos-Santos M. Early childhood caries and mutans streptococci: asystematic review. *Oral Health and Preventive Dentistry.* 2010; **8**: 59-70.

16. D'Amario M, Barone A, Marzo G, Giannoni M. Caries-risk assessment: the role of salivary tests. *Minerva Stomatology.* 2006; **55**: 449-463.

17. Neta T, Inokuchi R, Shinozaki-Kuwahara N, Kouno Y, Ikemi T, et al. Investigation of microbiological methods to estimating individual caries risk: evaluation of sampling methods and materials. *International Journal of Oral-Medical Sciences.* 2002; **1**: 29-32.

18. Hsu KLC, Osgood RC, Cutter GR, Childers NK. Variability of two plaque sampling methods in quantitation of *Streptococcus mutans*. *Caries Research.* 2010; **44**: 160-164.

19. Gotouda H, Shinozaki-Kuwahara N, Taguchi C, Ohta M, Shimosaka M, et al. Evaluation of dental caries and concomitant infection based on the ratio of cariogenic bacteria using plaque samples in adults. *Dentistry.* 2017; **7**: 461.

20. Gotouda H, Shinozaki-Kuwahara N, Taguchi C, Shimosaka M, Ohta M, et al. Evaluation of the proportion of cariogenic bacteria associated with dental caries. *Epidemiology (Sunnyvale).* 2017; **7**: 327.

21. Nishikawara F, Nomura Y, Imai S, Senda A, Hanada N. Evaluation of cariogenic bacteria. *European journal of Dentistry.* 2007; **1**: 31-39.

22. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology.* 2005; **43**: 5721-5732.

23. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, et al. Bacterial diversity in human subgingival plaque. *Journal of Bacteriology.* 2001; **183**: 3770-3783.

24. Greenstein G, Lamster IB. Bacterial transmission in periodontal diseases: a critical review. *Journal of Periodontology.* 1997; **68**: 421-431.

25. De Jong MH, Van Der Hoeven JS, Olijve JH. Growth of oral streptococcus species and Actinomyces viscosus in human saliva. *Applied and Environmental Microbiology.* 1984; **47**: 901-904.

26. Bowden GH. Does assessment of microbial composition of plaque/saliva allow for diagnosis of disease activity of individuals? *Community Dental and Oral Epidemiology.* 1997; **25**: 76-81.

27. Amerongen AV, Veerman EC. Saliva–the defender of the oral cavity. *Oral Disorders.* 2002; **8**: 12-22.

28. Filoche S, Wong L, Sissons CH. Oral biofilms: emerging concepts in microbial ecology. *Journal of Dental Research.* 2010; **89**: 8-18.

29. Lee SF, Li YH, Bowden GH. Detachment of Streptococcus mutans biolilm cells by an endogenous enzymic activity. *Infection and Immunity.* 1996; **64**: 1035-1038.

30. Asikainen S, Alaluusua S, Saxen L. Recovery of A. actinomycetemocomitans from teeth, tongue, and saliva. *Journal of Periodontology.* 1991; **62**: 203-206.

31. Umeda M, Contreras A, Chen C, Bakker I, Slots J. The utility of whole saliva to detect the oral presence of periodontopathic bacteria. *Journal of Periodontology.* 1998; **69**: 828-833.

32. Larmas M. Saliva and dental caries: Diagnostic tests for normal dental practice. *International Dental Journal.* 1992; **42**: 199-208.

33. Lenčová E, Broukal Z, Spížk J. Point-of-care salivary microbial tests for detection of cariogenic species-clinical relevance thereof-review. *Folia Microbiologica.* 2010; **55**: 559-568.