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
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 \((10^5 \text{ CFU/mL})\) (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 \((10^5 \text{ CFU/mL})\) (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>
<thead>
<tr>
<th>Sm/TS ratio (%) (Mean ± SD)</th>
<th>Plaque</th>
<th>Saliva</th>
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<tbody>
<tr>
<td>2.97 ± 5.91</td>
<td>0.86 ± 3.14</td>
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\[\text{Sm/TS ratio} = \frac{\text{S. mutans}}{\text{TS}}\]

Table 1. The mean number of S. mutans and the Sm/TS ratios (%) in plaque and saliva.
proportions of these bacteria in the plaque flora [14,32]. In this context, a significant correlation has been observed between the bacterial levels in plaque and saliva. Although distinct positive correlations between the salivary levels of cariogenic bacterial species reported between the salivary levels of MS and the plaque Sm/saliva Sm) were 20.56 ± 67.49 and 9.06 ± 15.95, demonstrating a significant difference between the two groups (p < 0.001; Wilcoxon signed-rank 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 samples (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 2).

**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 $8.01 \times 10^5$ CFU/mL with an average Sm/TS ratio of 0.86%, whereas the number of S. mutans detected in dental plaque was $24.33 \times 10^5$ 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