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Evaluation of Dental Caries and Concomitant Infection Based on the Ratio of Cariogenic Bacteria using Plaque Samples in Adults

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Abstract

Background: The aim of this study was to evaluate the association between dental caries and concomitant infections of *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) according to the ratio of cariogenic bacteria in plaque samples from adults to obtain basic data to develop clinical and chairside culture assay (kits) for assessment of dental caries risk.

Methods and Materials: Plaque samples from 192 adult volunteers (age range, 20-28 years) were obtained using sterile toothbrushes. Caries history and the number of decayed, missing, and filled (DMF) teeth were determined. Subjects were divided into high- and low-risk groups according to the ratio of *S. mutans* to total streptococci (Sm/TS ratio) and the number of *S. mutans*.

Results: The numbers of S. mutans and S. sobrinus and the Sm/TS ratio were significantly greater in the caries-active (CA) group than in the caries-free (CF) group. The number of bacteria and the Sm/TS ratio for the high-risk *S. mutans* plus detectable *S. sobrinus* subgroup was significantly greater in the CA group than in the CF group. High levels of *S. mutans* and *S. sobrinus* were significantly associated with dental caries. A significantly high caries risk was found between DMF teeth and the Sm/TS ratio for S. mutans when S. sobrinus was detected.

Conclusions: The number of dental caries was greater in adults with plaque samples concomitantly infected with high levels of *S. mutans* and *S. sobrinus* than in those with a single infection. These findings indicate the usefulness of analysis of cariogenic dental bacteria to detect adult populations with the severity of dental caries according to the Sm/TS ratio using dental plaque samples.

Keywords: Concomitant infection; Dental caries; Toothbrush plaque samples; Cariogenic bacteria ratio; Public health; Epidemiology

Introduction

Total Streptococci can be roughly grouped into the following four main species: *mutans streptococci (MS), Streptococcus mitis, Streptococcus salivarius, and Streptococcus sanguis*, which accounts for the majority of bacteria found in the mouth [1]. Among species that are generally termed MS, seven cariogenic streptococcal species, namely *Streptococcus mutans (S. mutans), Streptococcus sobrinus (S. sobrinus), Streptococcus rattus, Streptococcus cricetus, Streptococcus downei, Streptococcus macacae, and Streptococcus ferus*, are responsible for dental caries formation [2-7]. However, high caries activity has been shown to be more strongly associated with the prevalence of S. sobrinus than S. mutans [8-10]. Nonetheless, both species are considered important for the diagnosis and prevention of dental decay. Therefore, evaluation of the concomitant infection of S. mutans and S. sobrinus is necessary to assess the risk of dental caries with high precision.

Infants and children have been the subjects of most studies of dental caries formation [9-16], as reports of a single infection in adults [17,18] are scarce and no study of plaque samples has investigated concomitant infections in adults. Plaque, which harbors cariogenic bacteria, is an ideal sample for the assessment of caries activity [1,19]. However, as it is difficult to quantify plaque samples, paraffin- or chewing gum-stimulated saliva is more commonly used for the assessment of dental caries risk [2,4,5]. Among such sampling methods, the processing of plaque samples collected by brushing (plaque suspension) allows for quantitation and simple adjustments [20]. Therefore, plaque may be a clinically useful sample. However, this past study has focused on the reproducibility and validity of plaque samples, while none has investigated dental caries and the concomitant infection. Assessment of cariogenic bacteria levels is often used to determine the number of cariogenic bacteria [1-5]. However, few studies have reported the utility of the ratio of MS to total streptococci (TS) (MS/TS ratio) or *S. mutans* to TS (Sm/TS ratio) as an alternative approach to evaluate cariogenic bacteria [20,21]. Although the validity and reproducibility of these methods have been evaluated, an index

must have an association with dental caries to be a useful standard to assess the risk of dental caries [1-3]. To date, no study has evaluated the association of these ratios with dental caries.

It is difficult to obtain samples from children and elderly individuals with common methods that involve collection of saliva while chewing gum. Additionally, the use of plaque specimens is challenging owing to problems with quantitation and reproducibility, and it is much easier to obtain samples for quantitative plaque analysis by brushing. Simple commercially available culture kits have been used for clinical and chairside cultures, as well as clinical public health studies [1-3]. These kits can conveniently detect and estimate MS at chairside without the need for expensive equipment. However, these kits use saliva samples and only identify the seven bacterial strains of the MS group. Therefore, these kits cannot be used to specifically evaluate S. mutans, which is considered the most important pathogen in dental caries formation. An immunochromatographic assay that employs a monoclonal antibody to selectively detect *S. mutans* has been reported [2]. Although an immunohistochemistry kit was released commercially as an alternative to culture methods, it was later discontinued owing to imprecision and high cost. Molecular, biological, and epidemiological studies have demonstrated that *S. sobrinus* is a strong cariogenic species and infection of both S. mutans and S. sobrinus synergistically increases the risk of cariogenesis [2,4,7,22]. However, no simple assay (kit) is available for the easy detection of S. sobrinus.

The aim of this study was to evaluate the association between dental caries and concomitant infections of *S. mutans* and *S. sobrinus* according to the ratio of cariogenic bacteria from adults using plaque samples harvested from sterile toothbrushes. This pilot study obtained fundamental data for the development of a more precise clinical and chairside culture assay (kits) for assessment of dental caries risk, which employs quantitative plaque analysis and the Sm/TS ratio, without the use polymerase chain reaction (PCR) or immunohistochemical analysis.

Materials and Methods

Subjects and preparation of plaque simple

A total of 192 adult volunteers (age range, 20-28 years) in good physical condition with good oral health participated in this cross-sectional study conducted at the Nihon University School of Dentistry at Matsudo. Caries status was investigated and plaque samples were collected at this institution. The study protocol was approved by the Ethics Committee of the Nihon University School of Dentistry at Matsudo and all subjects signed an informed consent form after being informed about the aim of this study. Subjects with any systemic disease, using medications affecting salivary secretions, taking antibiotics were excluded from the study. The selected individuals were instructed not to eat/drink, use a mouth wash, or smoke 3 h prior to their appointment.Each subject was instructed to rinse the mouth, and then a large portion of plaque from all teeth was scraped off by vigorous brushing for 1 min using a sterile toothbrush, and was collected into a sterile bottle through a mouth rinse for 30s with 5ml phosphate-buffered saline and used as brushing-plaque sample [20].

Bacterial analysis

Mitis Salivarius agar plate (MS agar; Difco Laboratories, Inc., Detroit, MI, USA) and agar plate of Gold, [23] was used to culture

bacteria. Within 3 h after collection, the clinically isolated samples were dispersed by sonication (50 W, 20 s) using an ultrasonic apparatus (5202 Type; Ohtake Works Co., Ltd., Tokyo, Japan), serially diluted with chilled brain–heart infusion (BHI) broth. Laboratory strains and clinically isolated strains of S. mutans and S. sobrinus were cultured in BHI broth and incubated and inoculated on selective media using a spiral plating system and colony counter (Model–D; Gunze Sangyo, Inc., Tokyo, Japan). After anaerobic culture for 48 h, the number of TS colonies and MS colonies agar plates were counted, respectively. *S. mutans* and *S. sobrinus* were distinguished according to the colony morphology on agar plates, respectively. *S. mutans* and *S. sobrinus* on agar was determined by counting the number of respective colonies, and the ratio of S. mutans to total streptococci was designated as the Sm/TS ratio. A stereomicroscope was used to differentiate between *S. mutans* and *S. sobrinus* on the basis of the morphology of colonies formed with sucrose, the differences arising from how glucan is synthesized by the GTF enzymes formed outside or on the outer layer of the bacterial body about ratio of cariogenic bacteria. The Enzyme Linked Immunosorbent Assay (ELISA) was also used in cases where differentiation proved difficult [20,21].

Investigation of caries status and classification of caries risk groups

Caries status was determined according to the standards of the World Health Organization [24]. The number of decayed, missing, and filled (DMF) teeth was determined and recorded for each subject. The subjects were classified into either a high-risk or low-risk group according to the abundance of S. mutans and the Sm/TS ratio (in descending order).

Statistical analysis

The Mann–Whitney U test, χ2 test, and Fisher's exact probability test were used for comparisons between two groups. The Bonferroni test was used for comparisons among four groups. Descriptive statistics were assessed and all statistical analyses were performed using SPSS ver. 22.0 software (IBM Corp., Armonk, NY, USA). Data are presented as mean values ± standard deviation (SD).A probability (p) value <0.05 was considered statistically significant.

Results

The detection rate of TS in all subjects (n=192) was 100%. The detection rates of S. mutans and S. sobrinus were 92.2% and 21.9%, respectively (p<0.001, χ2 test, Figure 1). The mean (± standard deviation, SD) number of detected TS was $9.86 \pm 12.01 \times 10^7$ CFU/mL. The mean (± SD) numbers of detected *S. mutans* and *S. sobrinus* bacteria were 24.42 ± 78.93 and $2.84 \pm 19.79 \times 10^5$ CFU/mL, respectively. The mean Sm/TS ratio (%) was 2.21 ± 4.87%. The mean number of DMF teeth in all subjects was 7.89 ± 5.97. There were significant differences in the numbers of DMF teeth in subjects which had both *S. mutans* and *S. sobrinus* (11.07 ± 4.78) and DMF in subject with just *S. mutans* (7.40 ± 5.90), (p<0.01, Mann–Whitney U test).Subjects with ≥15 DMF teeth were included in the caries-active (CA) group (n=25) and those with no DMF teeth were included in the caries-free (CF) group (n=24). There were significantly greater numbers of *S. mutans* and *S. sobrinus* in the CA group than the CF group (p<0.001 and p<0.01), respectively. Additionally, the Sm/TS ratio (%) was significantly greater in the CA group (p<0.001, Mann–Whitney U test, Table 1).
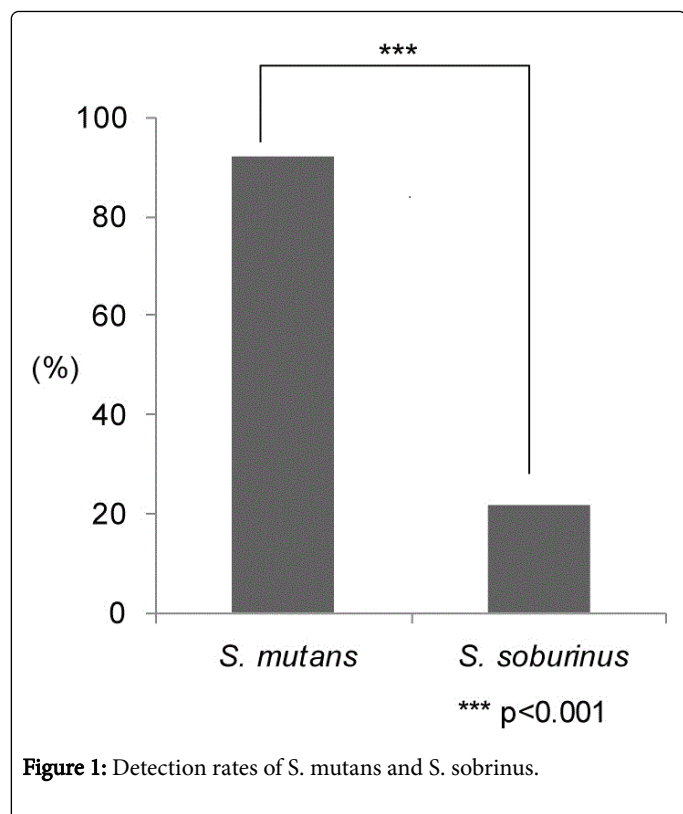
Figure 1: Detection rates of S. mutans and S. sobrinus.

	CA group (Mean ± SD)	CF group (Mean ± SD)	Significant Difference
Evaluation of numbers of cariogenic bacteria			
numbers of total streptococci(10^7 CFU/ml)	10.43 ± 10.85	11.88 ± 16.49	ns
numbers of *S. mutans* (10^5 CFU/ml)	40.39 ± 98.73	2.05 ± 3.42	***
numbers of *S. sobrinus* (10^5 CFU/ml)	6.01 ± 20.40	0.00043 ± 0.0021	**
Evaluation of the ratio of cariogenic bacteria			
Sm/TS ratio	(3.44 ± 3.53) × 10^{-2}	(0.55 ± 1.53) × 10^{-2}	***
**p<0.01; ***p<0.001			

Table 1: Numbers and ratios of cariogenic bacteria in the CA and CF groups.

In the evaluation of the ratio of cariogenic bacteria, there were significant differences in between the CA and CF groups in the rates of a high-risk Sm/TS ratio plus detectable *S. sobrinus* (Ratios-4G) (32.0% vs. 0.0%, respectively, p<0.01), the rates of a high-risk Sm/TS ratio plus non-detectable S. sobrinus(Ratios-3G)(60.0% vs. 20.8%, respectively, p<0.01), and the rates of a low-risk Sm/TS ratio plus non-detectable S. sobrinus(Ratios-1G) (4.0% vs. 75.0%, respectively, p<0.001). In the evaluation of the number of cariogenic bacteria, there were significant differences between the CA and CF groups in the rates of high-risk S. mutans plus detectable S. sobrinus (Numbers-4G) (32.0% vs. 4.2%, respectively, p<0.05) and the rates of low-risk S. mutans plus non-

detectable S. sobrinus (Numbers-1G) (20.0% vs. 75.0%, respectively, p<0.001, Fisher's exact probability test, Table 2).

There were also significant differences in the numbers of DMF teeth in Ratios-4G (n=25), Ratios-3G (n=71), Ratios-2G)(n=17), and Ratios-1G (n=79) subgroups (13.0 ± 4.3, 9.5 ± 6.6, 7.2 ± 4.4, and 5.0 ± 4.4, respectively, p<0.05, p<0.01, and p<0.001, respectively, Bonferroni test, Figure 2), as well as in the numbers of DMF teeth in Numbers-4G (n=26), Numbers-3G (n=70), Numbers-2G (n=16), and Numbers-1G (n=80) subgroups (12.4 ± 4.9, 8.5 ± 6.1, 7.8 ± 4.3, and 5.9 ± 5.6, respectively, (p<0.05 and p<0.001, respectively, Bonferroni test, Figure 3).

| Risk group	CA group (%)	CF group (%)	Significance
Evaluation of Ratios of cariogenic bacteria			
High S.m/TS + S.s	32	0	**
High S.m/TS + nonS.s	60	20.8	**
Low S.m/TS + S.s	4	4.2	ns
Low S.m/TS + nonS.s	4	75	***
Evaluation of numbers of cariogenic bacteria			
High S.m + S.s	32	4.2	*
High S.m + nonS.s	44	20.8	ns
Low S.m + S.s	4	0	ns
Low S.m + nonS.s	20	75	***
*p<0.05; **p<0.01; ***p<0.001			

Table 2: Rates of high- and low-risk S. mutans and ration of high- and low-risk S. mutans/total streptococci along with detectable and non-detectable S. sobrinus in the CA and CF groups.

Discussion

Cariogenic bacteria are transmitted and attach to the surfaces of the teeth of children after tooth eruption mainly via bacterial transfer from the mouths of close relatives (especially mothers) [1,4,25]. S. mutans and S. sobrinus are known to inhabit the human mouth at particularly high rates and are associated with dental caries formation [2-5]. The detected frequency varies, but S. mutans is detected in more than 90% of mouths, while S. sobrinus is detected in about 20%–30% by the culture technique [22]. The proportions of S. mutans and S. sobrinus detected in the mouths of adults in the present study were 92.2% and 21.9%, respectively, consistent with the proportions reported previously. In this study, for comparison with Simple culture kits utilized in clinical studies and at the chairside, these similar agar medium (culture medium) is used. According to this distribution, the assessment of the risk of dental caries is often solely based on the presence of *S. sobrinus* infection (irrespective of whether S. sobrinus is present), without performing bacterial count or ratio assessment [1,7,22]. The present study targeted S. mutans only to determine the ratio of cariogenic bacteria.
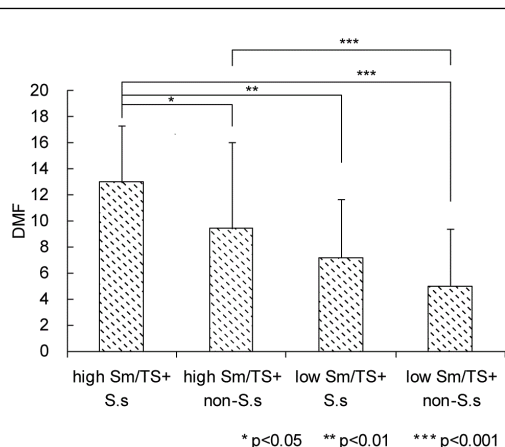
Figure 2: Numbers of decayed, missing, and filled (DMF) teeth in the high- and low-risk S. mutans/total streptococci ratio(Sm/TS) groups along with detectable and non-detectable S. sobrinus subgroups; Abbreviations: high Sm/TS, high-risk Sm/TS; low Sm/TS, low-risk Sm/TS; Ss, detectable S. sobrinus; non-Ss, non-detectable S. sobrinus.
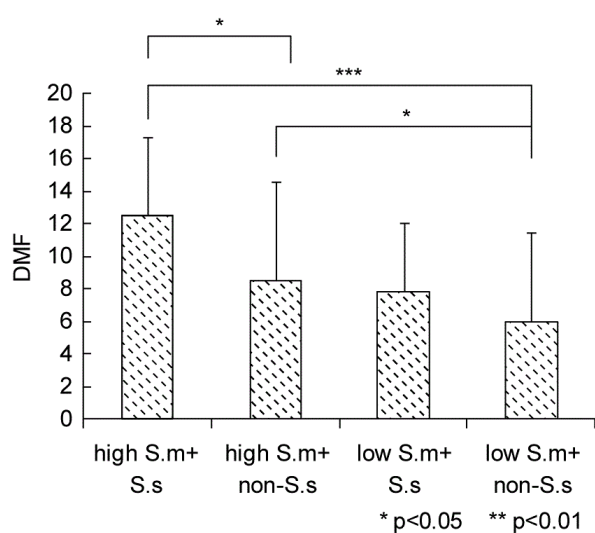
Figure 3: Numbers of decayed, missing, and filled (DMF) teeth in the high- and low-risk S. mutans numbers groups along with detectable and non-detectable *S. sobrinus* subgroups; Abbreviations: high Sm, high-risk S. mutans; low Sm, low-risk S. mutans; Ss, detectable S. sobrinus; non-Ss, non-detectable S. sobrinus.

Saliva stimulated by chewing has been used as a sample for the clinical detection and evaluation of the number of MS in the mouth, as it allows for quantitation [2]. Many dentists use simple culture kits to detect cariogenic bacteria with paraffin or chewing gum-stimulated saliva samples [2]. However, MS species are mainly found in plaque on the tooth surface [1]. Therefore, a bacterial count of plaque is required to clarify the caries-related activity of MS. A previous study that examined plaque collection methods to enable quantitation of bacteria revealed that plaque suspension after brushing teeth (brushing-plaque sample) is the best option [20]. In the present study, bacteriological test results were analyzed and evaluated using a culture method to quantitate *S. sobrinus* and *S. mutans* in brushing-plaque samples. In a previous study, the ratio of cariogenic bacteria obtained using a brushing-plaque sample was more reproducible than that obtained using a swabbing plaque sample [20]. The ratio of cariogenic bacteria in plaque was, on average, a few fold greater than that in saliva, indicating that plaque is a more useful sample than saliva [20]. However, the association between cariogenic bacteria in plaque with dental caries is a major requirement to consider a parameter appropriate for the assessment of caries activity [1-3]. Therefore, in the present study, the association between the ratio of cariogenic bacteria and dental caries in adults, and the usefulness of the brushing-plaque sample were assessed.

With regard to the associations of *S. mutans* and *S. sobrinus* with dental caries, *S. mutans* is isolated from dental caries more often than *S. sobrinus*, suggesting that *S. mutans* may be the major cariogenic Streptococcus species [26-29]. Furthermore, many epidemiological and in vitro studies have suggested that *S. sobrinus* is more strongly associated with the incidence of dental caries than *S. mutans* [2,4,7,22]. In the present study, the Sm/TS ratio was significantly greater in the CA group than in the CF group. Additionally, the proportion of subjects with concomitant infections of high-risk *S. mutans* and *S. sobrinus* was significantly greater in the CA group, while the proportion with low-risk *S. mutans* and non-detectable *S. sobrinus* was greater in the CF group. Evaluations of the bacterial ratio and numbers found differences in the proportion of concomitant infections and the numbers of DMF teeth between the CA and CF groups. Furthermore, the Sm/TS ratio was significantly greater in the high-risk *S. mutans* and non-detectable *S. sobrinus* subgroups of the CA group. Additionally, the Sm/TS ratio was significantly greater in the high-risk Sm/TS plus detectable *S. sobrinus* subgroup than in the low-risk Sm/TS plus detectable *S. sobrinus* subgroup, thereby confirming the usefulness of the Sm/TS ratio. Larger studies should be performed in the future to prospectively investigate the relationship between caries activity and the onset of caries formation. The results of this study confirmed the value of quantitative plaque assessment and caries ratio assay. Based on these initial results, a simple assay that involves culture for detecting *S. mutans* and *S. sobrinus* without immunohistochemical analysis or PCR is being developed.

Conclusion

The results of this study showed that the number of DMF teeth was greater in plaque samples of adults with concomitant infections of *S. mutans* (high levels) and *S. sobrinus* as compared with that in a single infection. These findings indicate the usefulness of analysis of cariogenic dental bacteria to detect adult populations with the severity of dental caries according to the Sm/TS ratio using dental plaque samples.

Competing Interests

The authors have no competing interest to declare.

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