the lesion depth (ld, μm) and mineral loss (ΔZ, vol%·μm), were measured. The data values were analyzed statistically by the repeated measure ANOVA followed by the Tukey-Kramer test for multiple comparison. The ld and ΔZ values are shown in Fig. 3 by individuals in types of gum. In all the individuals participating, significantly lower ld and ΔZ values were observed in the case of POs-Ca (+) gum indicating enhanced enamel remineralization. No remarkable mineral recovery was observed in the POs-Ca (−) gum group. The ld (means$\pm$SD=81 $\pm$15 μm) and ΔZ (2,825$\pm$593 vol%·μm) in the POs-Ca (+) gum group were significantly lower ($p<0.001$) compared with those after initial demineralization and in POs-Ca (−) gum group. The saliva secreted by chewing POs-Ca (+) gum had higher remineralization-enhancement activity than that from POs-Ca (−) gum. No difference was observed in volume, time-course change in pH level or contents of soluble P of saliva between chewing gum types or among volunteers. There were adequate amounts of P compared with Ca in saliva collected by chewing of the POs-Ca (−) gum. In the case of POs-Ca (+) gum, the Ca content was higher than that from chewing the POs-Ca (−) gum. The initial Ca/P ratio value in POs-Ca (+) gum-induced saliva was higher than that in POs-Ca (−) gum-induced saliva. The Ca/P ratio was 0.3 or less for POs-Ca (−) gum. These results suggested that remineralization enhanced by chewing the POs-Ca (+) gum was due to the increased soluble calcium in saliva that resulted in a higher Ca/P ratio value corresponding with the value (1.67) of hydroxyapatite.

2. *Intraoral evaluation.*

Based on the former results, we investigated the effects of the POs-Ca (+) gum on the remineralization of enamel *in situ*. Twelve healthy adult volunteers (6 males and 6 females; mean age, 21 years old) were randomly divided into 3 groups and participated in a double-blind intraoral study. In first, each volunteer wore a palatal appliance containing 3 demineralized enamel disks, and chewed one of the following experimental gums 4 times a day (after meals and before bed time) for up to 4 weeks.[18] The three groups were (i) the POs-Ca (−) gum group, (ii) the POs-Ca (+) gum group or (iii) sugar gum containing 62 wt% sucrose (the sucrose gum group). The chewing time was always 20 min and the palatal plate was preserved in the oral cavity for an additional 20 min. Except for the time of chewing the gum and the subsequent 20 min, the appliances were stored in a plastic container with 100% humidity. The results were that the remineralization rates (ld reduction percentage with respect to the mean ld value after initial demineralization) in the POs-Ca (+) group were about 67, 54 and 76% at the 1st, 2nd and 4th week, respectively. The remineralization rates in the POs-Ca (−) group ranged from 12 to 23%, being much lower than that in the POs-Ca (+) group. The sucrose group showed a negative value by the 4th week, indicating progression of demineralization. The remineralization rate of the POs-Ca (+) group was higher than that of the POs-Ca (−) group at the 1st, 2nd, and 4th week.[18] The present results were well consistent with the results of the *in vitro* evaluations with the HSI-test. Furthermore, the promoting of enamel remineralization of the POs-Ca (+) group was reconfirmed *in situ* by a two-week double-blind and cross-over design intraoral study.[25] The ld of the POs-Ca (+) group was significantly reduced by 51% compared with that in the sucrose group and by 44% compared with that in the POs-Ca (−) group (Fig. 4a). The ΔZ in the POs-Ca (+) group was also significantly lower by 30% compared with that in the sucrose group and by 25% compared with that in the POs-Ca (−) group (Fig. 4b). The effects of the gum on remineralization of dentin were also confirmed in a double-blind cross-over design intraoral study.[26]

Conclusion and perspective.

The promoted remineralization of enamel and dentin lesions by the POs-Ca (+) group can be explained as follows. The pH of the saliva during the chewing of the gums is estimated to increase from about 7 to 7.5. Since, in general, this relatively higher pH is not suitable for the solubilizing of Ca and phosphate, it is considered that POs-Ca in the saliva would aid to maintain the solubility of mineral ions even at pH 7–7.5 and, thereby, ionized Ca and P had potential to redeposit onto the residual hydroxyapatite crystals in enamel and dentin lesions. Thus, under the presence of POs, soluble Ca in saliva increases efficiently and, thereby, the salivary Ca/P ratio can increase nearly up to the rate of hydroxyapatite (1.67). In a former study, some ratios of Ca/P was investigated *in vitro*.[27] The Ca/P ratio of 1.67 showed significant reduction in ld and ΔZ of enamel lesion compared with other ratios. It was also suggested the elevation of the Ca/P ratio

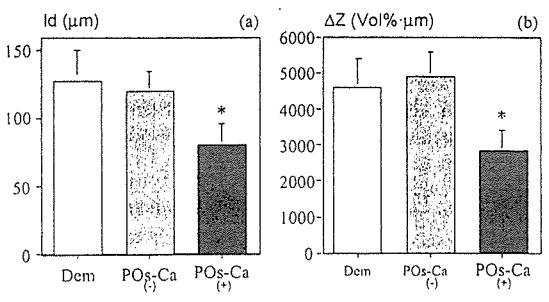

Fig. 3. Comparison of the lesion depth (a) and mineral-loss (b) values after salivary treatments.

Numbers of volunteers were 12 persons (6 males and 6 females; mean age=29.9 y old). Vertical bar, SD; $^*p<0.001$; Dem, initial demineralized enamel.

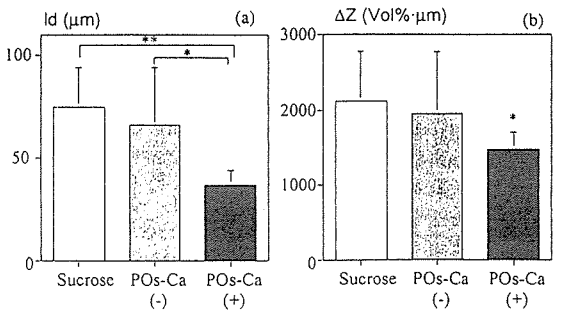

Fig. 4. Comparison of the lesion depth (a) and mineral loss (b) values after *in situ* experiments.

Numbers of volunteers were 12 persons (6 males and 6 females; mean age, 21 y old). Vertical bar, SD; $^*p<0.05$; $^{**}p<0.01$.

in saliva enhanced the remineralization. The gum containing POs-Ca would be able to compensate the remineralization effect of saliva itself. The results suggested that POs may be a novel and unique substance to enhance enamel remineralization, and could be utilized for caries prevention by a nutritional approach. It is generally known that a gastrointestinal disorder would occur when we take a food containing an excess amount of sugar alcohol at one time. We have already shown that the consumption of an excess amount of POs-Ca does not cause a gastrointestinal disorder.[28,29] We also confirmed that the orally administered POs-Ca was hydrolyzed and then absorbed completely in the small intestine in rats.[30] In conclusion, daily use of a sugar-free chewing gum containing POs-Ca can effectively enhance the remineralization both in enamel and dentin lesions. POs-Ca enhanced enamel remineralization by increasing the solubility of Ca in the oral environment and could be a beneficial material for oral health.

We thank Drs. Shigetaka Okada and Reiichiro Sakamoto for helpful suggestions through out the course of the study.

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馬鈴薯澱粉由来リン酸化オリゴ糖カルシウムのオーラルヘルスへの応用

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澱粉には，その構成糖にリン酸基がエステル結合している糖を含むことが知られている．著者らは馬鈴薯澱粉の加水分解物より，リン酸基がエステル結合している糖，つまり，リン酸化オリゴ糖カルシウム（POs-Ca）を調製してきた．このリン酸化オリゴ糖は二つの画分 PO-1 画分および PO-2 画分から構成されていた．PO-1 画分はリン酸化オリゴ糖の主な成分であって，マルトトライオース，マルトテトラオース，およびマルトペンタオースから構成されており，分子内に 1 個のリン酸基を有していた．PO-2 画分は主にマルトペンタオースおよびマルトヘキサオースから構成されており，少なくとも 2 個のリン酸基を分子内に有していた．リン酸化オリゴ糖はカルシウムと水溶性の複合体を形成し，カルシウム-リン酸の沈澱形成を阻害する効果を有していた．以上の結果をもとにリン酸化オリゴ糖のカルシウム塩（POs-Ca）を食品素材として開発してきた．POs-Ca は，水溶性カルシウム供給のための食品素材として優れていた．また，う蝕予防の観点から，リン酸化オリゴ糖はう蝕原因細菌であるミュータンス連鎖球菌の栄養源にならず，本菌の産生する酸によるプラーク内の pH の低下も抑制する作用を有していることを明らかにした．さらに，POs-Ca は初期う蝕を誘発したエナメル質の再石灰化を効果的に促進する作用も有していることがわかった．ここでは，POs-Ca を関与成分としたシュガーレスガムの初期う蝕の再石灰化効果を明らかにし，POs-Ca の口腔保健への応用開発について紹介する．

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〔質　問〕　信州大　北畑

1）再石灰化率から見ますと POs-Ca の濃度が低い方が良いということでしょうか？

2）ガムを噛んで 10 分後，20 分後で Ca/P 濃度比を比較すると，10 分で十分ではないでしょうか？

〔答〕

1）再石灰化には試験溶液中の Ca/P 濃度比が大きく影響します．*In vitro* の試験系において，POs-Ca を高濃度添加しますと Ca/P 濃度比が高値になり，再石灰化率が低下するものと考えております．よって，Ca/P 濃度比を考慮した POs-Ca の利用が重要です．つまり実際の唾液には唾液用の POs-Ca 濃度が有効ということです．

2）個人差はありますが，ガムの殆どの成分は約 10 分間で唾液中に溶出するものと考えられます．POs-Ca も 10 分間で殆ど溶出してきておりました．再石灰化効果に関しても 10 分間の咀嚼で効果が得られることも確かめております．このことから，ご質問のように 10 分間の咀嚼で十分効果が発揮されることも考えられます．一方，唾液量を考えた場合，10 分間の咀嚼で約 20 mL の唾液量になり，20 分間の咀嚼では約 30 mL の量に増加します．唾液には再石灰化のみではなく，口腔機能維持にとって様々な重要な役割を担っております．総合的に考え，20 分程度のガムの咀嚼を推奨しております．

〔質　問〕　食総研　山本

1）POs は口腔内のアミラーゼによってどのような影響を受けるか？

2）Ca を結合して可溶化する物質には POs 以外にも CPP(カゼインホスホペプチド)，クエン酸，ポリグルタミン酸など様々あるが，POs がそれらに比べて優れている点はどんなところか？

3）歯の再石灰化の際には，アモルファスリン酸カルシウム(ACP)から出発して結晶転移を経てハイドロキシアパタイト(HA_p)になるが，Ca/P の最適値は HA_p の 1.67 で良いか．ACP や第二リン酸カルシウム(DCPD)の Ca/P でなくても良いか？

〔答〕

1）POs は α-アミラーゼを馬鈴薯澱粉に作用させて得られてきた産物ですので，これ以上の加水分解作用は唾液中のアミラーゼでも受け難いと考えております．私たちのこれまでの研究でもヒト唾液由来 α-アミラーゼの POs への作用特性を詳細に報告しております．

2）再石灰化とは，次の二つのプロセスで進むと考えられます．①歯の脱灰患部にカルシウムイオンとリン酸イオンが供給される．②供給されたカルシウムイオンとリン酸イオンが脱灰患部の結晶成長に使用される．その観点から，再石灰化促進物質とは，①カルシウム-リン酸の不溶化を抑制するが，②脱灰患部の結晶成長を助長するという機能が必要と考えられます．本観点からカルシウムイオンとのイオン結合能力が重要な問題になります．分子内に過剰の結合リン酸基が存在する場合には，キレート能力が高すぎて，結晶成長を阻害することも確認してきております．ただし，詳細な情報を得ることは今後の課題と考えております．

3）HA_p が形成されるまでには，DCPD，トリカルシウムホスフェイト(TCP)およびオクタカルシウム(OCP)などが関与していることが知られております．POs-Ca を咀嚼した際の唾液中の Ca/P 比 1.67 を目標にしておりますが，ガム食品の性質上，咀嚼開始直後から経時的に Ca/P 比は低下してゆきます．そして，10 分以降には通常の唾液組成の Ca/P 比 0.3 程度に戻ってしまいます．一般的に唾液に再石灰化の機能が備わっていることは周知の事実であることから，必ずしも Ca/P 比 1.6 でなくとも再石灰化は生じます．しかし，短期間での効果的な再石灰化を生じさせるためには，Ca/P 比を 1.67 にすることであると考えております．

Oral Microbiology Immunology 2004: 19: 386–389
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Oral Microbiology and Immunology

Molecular analysis of age-related changes of *Streptococcus anginosus* group and *Streptococcus mitis* in saliva

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Morita E, Narikiyo M, Nishimura E, Yano A, Tanabe C, Sasaki H, Hanada N. Molecular analysis of age-related changes of Streptococcus anginosus *group and* Streptococcus mitis *in saliva.*
Oral Microbiol Immunol 2004: 19: 386–389. © Blackwell Munksgaard, 2004.

The purpose of this study was to survey the prevalence of streptococcal species, especially *Streptococcus anginosus* (which has been reported to be associated with cancer in the upper digestive tract), *Streptococcus constellatus*, and *Streptococcus intermedius* in the saliva of different age groups. A sequence analysis of 16S rDNA was performed and DNA quantified using real-time polymerase chain reaction. The *S. anginosus* level increased with age, whereas the levels of *S. constellatus* and *S. intermedius* did not change. *Streptococcus mitis* was the predominant species in the saliva of all the age groups but, unlike the *S. anginosus*, the proportion of *S. mitis* in the salivary bacteria decreased with age. The increase in *S. anginosus* with age should be carefully monitored because of its association with diseases, including cancer.

Key words: real-time polymerase chain reaction; saliva; *Streptococcus anginosus*; *Streptococcus mitis*

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Accepted for publication July 1, 2004

Streptococcus anginosus is a currently recognized species of the '*Streptococcus milleri*' group, the name used for heterogeneous oral streptococcal strains associated with purulent infections (14). The *S. milleri* group comprises at least three different species: *S. anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* (14). Awareness of the clinical importance of *S. anginosus* has gradually increased because several studies have reported a close association between *S. anginosus* infection and cancer in the upper digestive tract (3, 7, 8, 12). In spite of this clinical significance, information on the prevalence of *S. anginosus* in the oral cavity is limited because most studies on this subject were performed before the current classification criteria of the *S. anginosus* group was established (11). Recent analyses using real-time polymerase chain reaction (PCR) found extremely low levels of *S. anginosus* in the saliva (2, 10). However, these studies did not consider the variety of *S. anginosus* strains. Nor was age considered, although microbial infection in the oral cavity appears to change with age (5). It was reported that the streptococcal salivary colony-forming units were higher in adults than in children, and that the isolation frequency and proportion of streptococcal species change with age (11). The incidence of cancer is highest in people in their sixties (1). The oral cavity may act as a reservoir of *S. anginosus*, a potential pathogen, and saliva is the most probable carrier. The emphasis of this study was the molecular analysis of subsets of the salivary microbiota in different age groups, focusing on *S. anginosus*.

Material and methods

Saliva samples

Saliva samples were obtained from systemically healthy volunteers aged 25–70 years. These samples were centrifuged, then frozen and stored at −80°C until use. Written informed consent was obtained from all the volunteers.

Bacterial strains

S. anginosus ATCC 33397, *S. intermedius* ATCC 27335, *S. constellatus* ATCC 27823, *Streptococcus mutans* LM 7, *Streptococcus sobrinus* AHT, *Streptococcus sanguinis* ATCC 10556, *Streptococcus gordonii*

Table 1. S. anginosus, S. constellatus, and *S. intermedius* strains and accession numbers for the DDBJ/EMBL/GenBank nucleotide sequence databases from which DNA sequence data were used for designing specific primers

Species/strains	Accession numbers
S. anginosus strain ATCC33397	AF352808
S. anginosus genotype VA8466	AF306838
S. anginosus strain 920	AF145246
S. anginosus strain 1007	AF145245
S. anginosus strain 414	AF145243
S. anginosus strain 21	AF145242
S. anginosus strain 1204	AF145240
S. anginosus strain 367	AF145239
S. anginosus strain GTC822	AB006121
S. anginosus strain GTC821	AB006120
S. constellatus strain 1259	AY277942
S. constellatus strain 919	AY277941
S. constellatus strain 1192	AY277940
S. constellatus strain 15	AY277939
S. constellatus strain 857	AY277938
S. constellatus strain 1	AY277937
S. constellatus strain ATCC27823	AF104676
S. constellatus strain 206	AF104677
S. constellatus strain VAMC3868	AF169356
S. constellatus strain VAMC5464	AF169353
S. intermedius strain 488	AF104673
S. intermedius strain 125	AF104672
S. intermedius strain 535	AF104674
S. intermedius strain ATCC27335	AF104671
S. intermedius strain B33	AJ491836

ATCC 10558, *Streptococcus mitis* ATCC 6249, and *Streptococcus salivarius* ATCC 9759 were cultured.

DNA extraction

Genomic DNA was isolated from saliva and bacteria by a standard phenol–chloroform method. DNA content was determined spectrophotometrically.

PCR cloning and sequence analysis

Equal amounts of DNA extracted from the saliva of 10 people were mixed for each age group (25–49-year-old group, 50–69-year-old group and 70-year-old group) and used as templates. For amplification of a portion of the 16S rDNA gene of many oral bacteria from saliva, PCR was performed with primers Ust1X and Ust2E (Tables 1 and 2), and PCR cloning and sequence analysis of 16S rDNA were performed as described previously (4). A species was determined when its sequence had greater than 90% homology to bacteria.

Alignment and primers

16S rDNA sequences of 10 strains of *S. anginosus*, 10 strains of *S. constellatus*, 5 strains of *S. intermedius* (Table 1) and other streptococcal species were aligned by Clustal W (13) to design specific primers for amplification of 16S rDNA of *S. anginosus*, *S. constellatus*, and *S. intermedius*. Primers Ust1 and Ust2 modified from Ust1X and Ust2E were used for amplification of the 16S rDNA gene of many oral bacteria (Table 2).

Quantitative real-time PCR

Real-time PCR was performed on the ABI Prism Sequence detection System 7700 (Applied Biosystems, Foster City, CA) using SYBR green chemistry. The reaction mixture in a total volume of 25 µl contained SYBR Green Core Regent (Applied Biosystems), 3 mM $MgCl_2$, 200 nM of each primer, and 5 µl of DNA solution. The reaction was started with an incubation of 2 min at 50°C, followed by 10 min at 95°C, then 50 cycles of 15 s at 95°C and 1 min at 68°C.

Statistical analysis

Differences in the levels of *S. anginosus*, *S. constellatus*, and *S. intermedius* DNA in the three age groups were statistically analyzed using the Mann–Whitney *U*-test.

Results

Distribution of *Streptococcus* in saliva

The diversity of the bacterial flora in saliva was examined in three age groups. Table 3 describes species that had more than 90% similarity to partial sequences of 16S rDNA obtained for clones of salivary DNA. In all, 119 of 192 clones were identified as *Streptococcus*. *Streptococcus* accounted for 93% of identified strains in 25–49-year-olds, 45% in 50–69-year-olds, and 58% in 70-year-olds. *S. mitis* was the most frequently detected species in all age groups, and the proportion decreased with increasing in age. *S. mitis* accounted for 76% of *Streptococcus* in subjects aged 25–49 years, 46% in 50–69-year-olds, and 43% in 70-year-olds. Diversity of bacterial species increased as age increased. *S. anginosus* was not detected in any age group. *S. constellatus* was the only species detected among the *S. anginosus* group.

Table 2. Primers used in this study

Primer	Purpose	Bacterial specificity	Sequence	Position[a]
F13	Real-time PCR	*S. anginosus*	CTAATACATGCAAGTAGG	48
F6	Real-time PCR	*S. anginosus*	CAAGTAGGACGCACAGTT	58
F8	Real-time PCR	*S. anginosus*	CAAGTAGGACGCACAGTC	58
R3	Real-time PCR	*S. anginosus*	CAAGCATCTAACATGTGTTAC	186
ConF2	Real-time PCR	*S. constellatus* (*S. intermedius*)	CACCGTAGTTTACTACACCGTATT	78
ConR4	Real-time PCR	*S. constellatus* (*S. intermedius*)	CTACCATGCAGTAAATGTTC	181
Ust1	Real-time PCR	Oral bacteria	GAACGGGTGAGTAACGCGTAGGT	106
Ust2	Real-time PCR	Oral bacteria	CACTCACGCGGCGTTGCTCGGTC	387
Ust1X	PCR cloning	Oral bacteria	GCTCTAGAGAACGGGTGAGTAACGCGTAGGT	106
Ust2E	PCR cloning	Oral bacteria	GGAATTCCACTCACGCGGCGTTGCTCGGTC	387

[a]The 5′ position in *Escherichia coli* 16S rDNA numbering convention.

Table 3. Bacterial species inferred from PCR cloning analysis of the saliva of healthy people in the three age groups

Species	No. of clones			
	25–49 years	50–69 years	70 years	Total
S. mitis	32	13	21	66
S. salivarius	2	0	7	9
S. infantis	3	3	6	12
S. sanguinis	1	4	2	7
S. parasanguinis	0	4	4	8
S. australis	0	2	5	7
S. cristatus	1	1	2	4
S. constellatus	0	1	0	1
S. anginosus	0	0	0	0
Unidentified *Streptococcus*	3	0	2	5
Other bacterium	3	34	36	73

Primer design

An alignment of *S. anginosus*, *S. intermedius*, *S. constellatus*, and other *Streptococcus* revealed a variety of *S. anginosus* strains in 16S rDNA (Fig. 1). In previous studies, a variable region was used as primer to detect *S. anginosus* (2, 3, 10). Three different forward primers were designed to determine if this variable region is suitable as an *S. anginosus*-specific primer (Fig. 1, Table 2). F6 and F8 primers are highly specific but include the variable region used for detecting *S. anginosus* in former studies (2, 3, 10). F13 primer is less specific but does not include this variable region. In order to maintain specificity to *S. anginosus*, these three forward primers were used with a specific reverse primer, R3 (Fig. 1, Table 2). The alignment also revealed a strong similarity between *S. intermedius* and *S. constellatus*, making it difficult to design specific primers to distinguish *S. constellatus* from *S. intermedius*. Therefore, a primer set (ConF2, ConR4) that detected both *S. constellatus* and *S. intermedius* was designed (Table 2).

Quantification system of *S. anginosus*, *S. constellatus*, and *S. intermedius*

Real-time PCR was used to analyze DNA extracted from *S. anginosus*, *S. constellatus*, *S. intermedius*, *S. mitis*, *S. gordonii*, *S. salivarius*, *S. mutans*, *S. sanguinis*, *and S. sobrinus* in order to examine quantification systems with new primer sets of *S. anginosus* and *S. constellatus* (*S. intermedius*). When 10^1–10^6 fg DNA from *S. anginosus* was added in serial dilutions to the reaction mixture for real-time PCR systems of *S. anginosus*, detection and quantification were linear over the range of the DNA concentration examined. The real-time PCR system of *S. constellatus* (*S. intermedius*) was examined in the same way, and a similar result was obtained. When 10^4 fg of DNA from other species were assayed, the calculated values

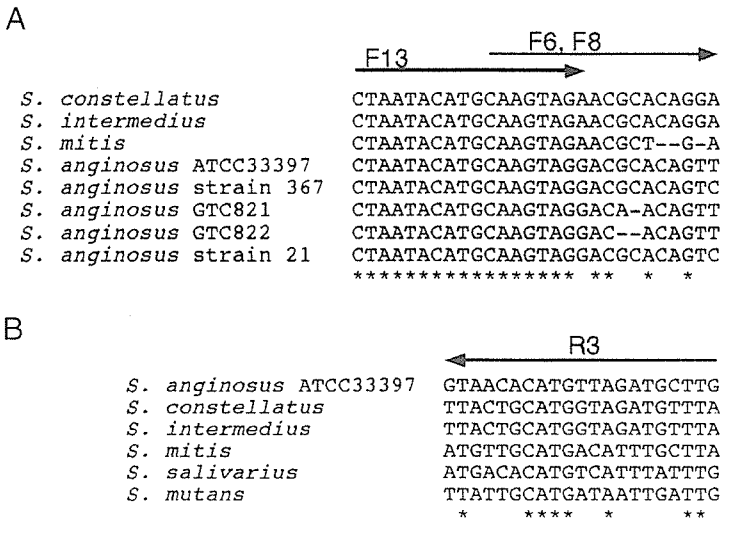

Fig. 1. Alignments of 16SrDNA of streptococcal species and *S. anginosus* strains. Arrows indicate primers. Stars represent identical nucleotides.

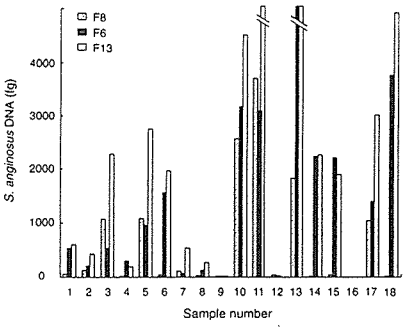

Fig. 2. Quantified values of *S. anginosus* DNA in 50–69-year-olds obtained using three different systems of real-time PCR. Ten ng of salivary DNA was used.

derived from a standard curve were lower than the detectable level, 10^1 fg. It was therefore concluded that each system was specific for each species. Three *S. anginosus* forward primers were then examined to determine whether they were suitable for the *S. anginosus* quantification system. *S. anginosus* DNA in saliva samples was quantified, but the quantified values derived from each system were different. Figure 2 depicts quantified values of *S. anginosus* in the saliva from the 50–69-year-old group. The system using F6 and F8 primers often exhibited completely different values from one another (6 of 18 samples showed more than 10 times difference), and they were not proportional. The system using the F13 primer usually had the highest values, which were comparatively close to the second highest values. As these results suggested that F6 and F8 primers are too specific to quantify various types of *S. anginosus* present in the oral cavity, F13 was chosen as the primer to quantify *S. anginosus* in the present study.

Quantification of *S. anginosus* and *S. constellatus* in saliva

The quantities of *S. anginosus*, *S. constellatus* (*S. intermedius*), and oral bacteria in saliva samples from 65 healthy people aged 25–70 years were determined, and the proportions of *S. anginosus* and *S. constellatus* (*S. intermedius*) in oral bacteria were calculated (Table 4). Results demonstrated that the average proportion of *S. anginosus* in oral bacteria increased with age: 0.38% at 25–49 years of age, 1.12% at 50–69 years, and 2.02% at 70 years. Statistical analysis indicated that the level was significantly higher at age 70 than at ages 25–49. Additionally, the oral bacteria in the saliva of 25% of 50–69-year-olds contained more than 2% *S. anginosus*, whereas the highest

Table 4. Average proportion of *S. anginosus* or *S. constellatus* (*S. intermedius*) to oral bacteria in the three age groups

	Average age	Sample no.	*S. anginosus* (%)	*S. constellatus* (*S. intermedius*) (%)
25–49 years	33.2	17	0.38 ± 0.32	0.06 ± 0.04
50–69 years	58.3	18	1.12 ± 1.74	0.11 ± 0.26
70 years	70	30	2.02 ± 3.49	0.07 ± 0.09

level found in the 25–49-year-old group was only 1.7% (data not shown). In contrast, the average ratio of *S. constellatus* (*S. intermedius*) to oral bacteria was about 10–40 times lower than that of *S. anginosus*, with no significant difference between age groups.

Discussion

Previous studies demonstrated very low levels of *S. anginosus* in saliva (2). In the present study, the distribution of streptococcal species in the saliva of healthy people was determined by clonal analysis of 16S rDNA sequence. It was demonstrated that *S. anginosus* as well as other *S. milleri* group species, *S. constellatus* and *S. intermedius* were minor species in all three age groups. However, quantification of *S. anginosus* DNA demonstrated that most people possess *S. anginosus* to some extent in their saliva. These results were obtained with the use of our new primer.

The average level of *S. anginosus* in the oral bacteria increased with age, whereas the average level of *S. constellatus* (*S. intermedius*) did not change and the proportion of *S. mitis* in the oral bacteria decreased. *S. mitis* was the predominant oral streptococcal species in infants, both in its prevalence and in its proportion of the oral streptococci. It may thus be the major component of the initially colonizing streptococcal microbiota of infants (9). The trend toward a decreasing proportion of *S. mitis* might start from an early age. Viridans group streptococci, including *S. mitis,* are known to induce inflammation of renal tissue (15). However, at least in the oral cavity, a prevalence of *S. mitis* may indicate young healthy microflora. In contrast, *S. anginosus*, which increased with increasing age in inverse proportion to *S. mitis*, should be carefully monitored due to its association with various kinds of infectious diseases such as endocarditis and cancer in the upper digestive tract.

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A survey on the risk factors for the prevalence of dental caries among preschool children in Japan

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Reprinted from

PEDIATRIC DENTAL JOURNAL 14(1): 79–85, 2004

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Abstract The etiology of dental caries has been suggested to be multifactorial. We conducted a cross-sectional study to investigate the contribution of the risk factors for dental caries, surveying a total of 645 preschool children at medical check-ups. Among 10 factors investigated in this study, salivary flow, amount of Lactobacillus, amount of total Streptococci, amount of mutans streptococci, and daily number of times of sweet soft drinks correlate with the prevalence of dental caries. Multivariate Logistic regression analysis using the five factors that correlated produced only two factors, salivary levels of mutans streptococci and Lactobacillus, which correlated with the prevalence of dental caries. Furthermore, clear dose-response relationships were observed in these two factors. We therefore suggest that cariogenic bacteria are the most important risk factor for dental caries among preschool children in Japan.

Key words
Lactobacilli,
Mutans streptococci,
Preschool children,
Risk factors,
Saliva

Introduction

Dental caries has been suggested to be a multifactorial disease. Keyes has proposed three main factors in the etiology of dental caries: host, substrate, and microflora[1]. Furthermore, the mechanism of dental caries was theoretically explained[2]. Some clinical trials have shown that controlling these factors suppresses the incidence of dental caries. For the host factors, sodium fluoride has been suggested as useful for preventing dental caries both clinically and economically[3]. For the substitute factors, diet sugar consumption is correlated with the incidence of new dental caries[4,5], and restricting the intake of diet sugar has been suggested to reduce the incidence of dental caries[6]. Recently, xylitol has been shown to be useful as an alternative sucrose, and its efficiency was confirmed[7]. For microflora factors, plaque control has conventionally been used as the primary preventive method. Many clinicians have used plaque control as a tool for fighting both dental caries and also periodontal disease. Furthermore, application of anti-microbial drugs could reduce the number of mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) in saliva or plaque for a few months[8].

These factors are all correlated with the etiology of dental caries. However, the weight of these factors contributing to the prevalence or incidence of dental caries has not been clarified. These factors may confound each other in attempts to explain the etiology of dental caries, and some studies have shown that controlling only one of these factors could not suppress the incidence of dental caries completely.

In this study, we obtained clinical samples and information about the etiology of dental caries by questionnaires at preschool medical check-ups. The aim of this study was to analyze the contribution

Received on September 22, 2003
Accepted on January 14, 2004

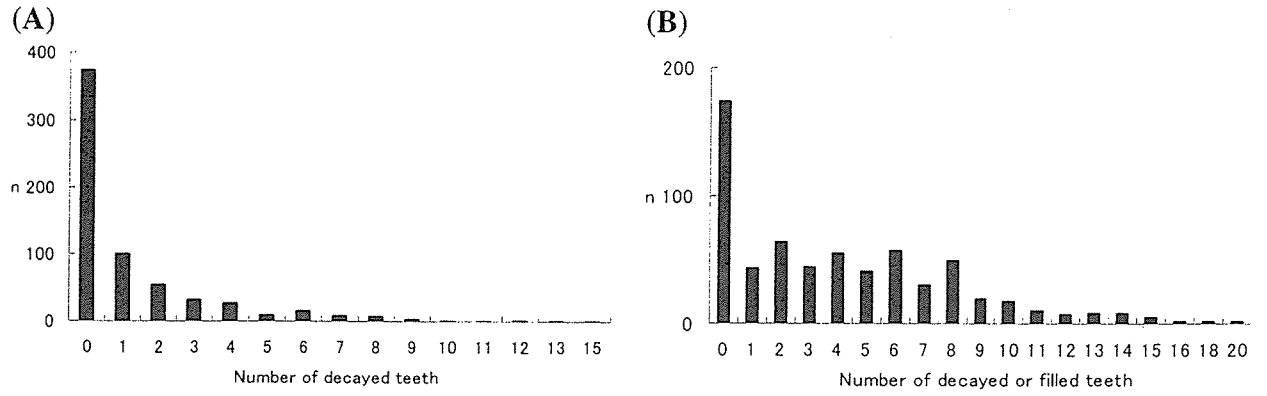

Fig. 1 The distribution of dt and dft

The distribution of the number of decayed deciduous teeth (A) and decayed or filled teeth (B) in this study. The median of the decayed teeth was 0 and decayed or filled teeth was 3. With the erupted permanent teeth, no decayed of filled teeth was observed.

of these factors in children by multiple Logistic regression analysis to clarify their importance.

Materials and methods

Study population

The study population was sampled from pre-elementary school children (five or six years old) residing in the Ena and Nakatsugawa areas of Gifu Prefecture, Japan. The fluoride concentration of drinking water in this area is less than 0.8 ppm. Thirteen of the thirty elementary schools, 9 from Ena area and 4 from Nakatsugawa, were selected to sample the population. Children were informed of the survey by the letter from the municipal office announcing their entry into elementary school and a total of 645 children participated in this study. Twelve children dropped out, primarily because of relocation and missing check ups due to illness. We obtained clinical samples and questionnaires during the pre-school medical check-ups; we obtained informed consent at the collection of the questionnaires.

Clinical examination and clinical samples

Dentists conducted oral examinations under a light and using dental mirrors. Teeth examined for dental caries were scored as sound, decayed or filled. The decayed or filled teeth were identified according to the WHO standard method and criteria[9].

Saliva samples were obtained by having subjects chew a gum base that contains no taste or flavor additives for 3 mins. The 3-min stimulated salivary flow and salival buffering capacity were evaluated by pH testing paper (Toyoroshi, Tokyo, Japan).

Microbial procedures

To quantify the total Streptococci, mutans streptococci, and Lactobacilli in saliva, we performed microbial procedures according to the method described previously[10]. Saliva samples (50μl) were sonicated by ultrasonic dispersion (60 power output) for 10 seconds and poured onto Mitis-Salivarius agar (MS, Gibco, Tokyo, Japan) plates for total Streptococci, improved Mitis-Salivarius agar plates containing 0.02 M bacitracin (Wako, Osaka, Japan) (MSB) and 2 ug/ml of Gramidine[11] for mutans streptococci and Rogosa SL agar plates (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) for Lactobacilli using an EDDY JET spiral system (Gunze Sangyo, Inc., Tokyo, Japan). In the improved MSB plate, the growth of the mutans streptococci are higher than that in the conventional MSB plate[12]. All samples were then incubated for 48 hours anaerobically. After the anaerobic incubation, we counted the colonies on each agar plate and calculated the number of bacteria per ml whole-saliva.

Questionnaires

Questionnaires were distributed by mail with the announcement of school participation and collected at preschool medical check-ups. The questionnaires consisted of five items concerning fluoride usage and diet. Fluoride usage was evaluated by daily usage of fluoride containing dentifrices (yes or no), the experience of fluoride varnish at private dental office or usual health care check-ups (yes, experienced or never) and daily use of mouthwash with fluoride (yes, experienced or never). The questionnaire on

Table 1 Descriptive analysis of each factors

	This study		National average in Japan
	Mean	SD	
Number of decayed teeth	1.25	2.19	2.02
Number of filled teeth	2.90	3.26	2.37
Number of df teeth	4.15	4.01	4.38
Salivary flow (ml/3 mins)	2.96	1.74	—
Salivary pH	7.276	0.198	—
Total Streptococci (CFU/ml, $\log_{10}$ count)	6.887	0.261	—
Lactobacillus (CFU/ml, $\log_{10}$ count)	2.561	2.35	—
mutans streptococci (CFU/ml, $\log_{10}$ count)	4.208	2.305	—

Mean and standard deviation of the data from the oral examination and bacterial cultures obtained in this study. The mean number of decayed or filled teeth was below the average of a national survey in Japan. The data of the national average of Japan were obtained from The Survey of Dental Diseases by Health Policy Bureau Ministry of Health and Welfare Japan (1999).

diet sugar intake consisted of two items, the number of daily intakes of sweet juice and the daily intake of sweet snacks (once, twice, three times or more than four times).

Statistical analysis

Before the analysis, patients were divided into two groups: subjects free from dental caries and subjects with at least one decayed or filled tooth (df teeth). For the high risk children, subjects were divided into two groups by the 75th percentile of the distribution by of the df teeth. As with microbiological factors, the bacteriological counts were $\log_{10}$-transformed prior to statistical analysis to normalize the variances. After evaluation of the distribution, amount of the mutans streptococci and Lactobacillus were categorized into four groups by the 25th, 50th and 75th percentile of the distribution.

Logistic regression analysis was used to evaluate the crude or adjusted odds ratios and their associated 95 percent confidence intervals. To eliminate the confounding factors, multiple Logistic regression analysis was used for factors correlated to the prevalence of dental caries. To confirm the dose-response relationships, the final factors correlated with the prevalence of the dental caries were classified according to the distribution.

Then two-way ANOVA was used to investigate the co-effect for the mutans streptococci and Lactobacillus for the df teeth.

Results

Forty-two percent of the children participating in this study had decayed teeth, and 73.1% had df teeth. The distribution of results is shown in Fig. 1. Table 1 shows the mean and SD of the number of the decayed, filled, and df (decayed or filled) teeth, and the salivary flow, salivary pH and salivary levels of the bacteria investigated in this study. Table 2 shows the categorized results from questionnaires. Salivary levels of mutans streptococci were not detected

Table 2 Results of the data obtained from questionnaires in this study

	n	%
Usage of fluoride containing dentifrice		
Yes	404	61.1%
No	245	37.1%
Experience of fluoride varnish		
Regularly	382	57.8%
Experienced	235	35.6%
Never	33	5.0%
Experience of fluoride mouth rinse		
Regularly	38	5.7%
Experienced	108	16.3%
Never	495	74.9%
Sweet soft drink intake (daily)		
Once	325	49.2%
Twice	228	34.5%
Three times	79	12.0%
Four or more times	17	2.6%
Sweet snack intakes (daily)		
Once	34	5.1%
Twice	393	59.5%
Three times	198	30.3%
Four or more times	24	3.7%

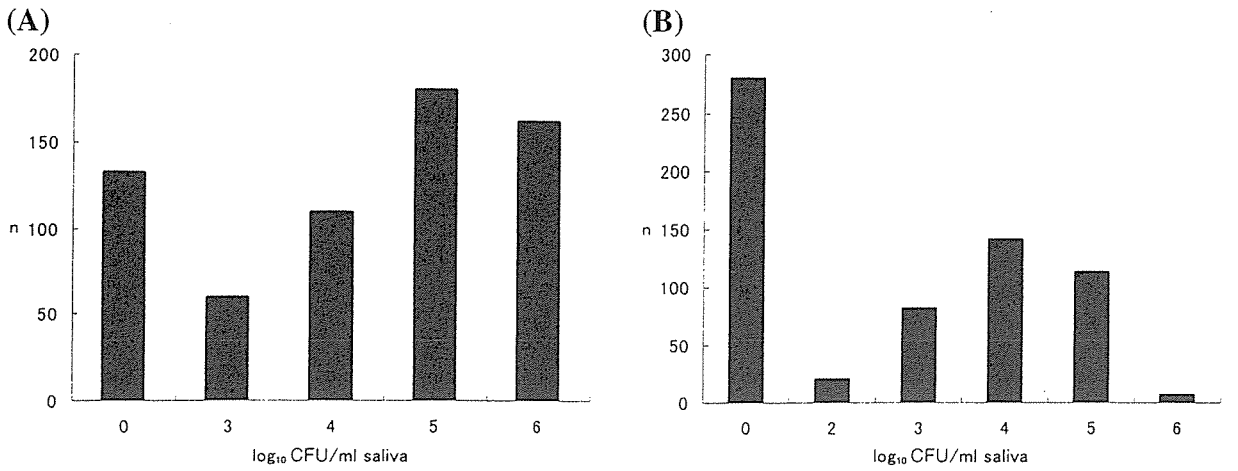

Fig. 2 The distribution of the microflora

The distribution of the mutans streptococci (A) and Lactobacillus (B) in this study. 20.6% of children could not detected mutans streptococci from their saliva, however, from 25.1% of children, mutans streptococci could be detected more than 10^6 CFU/m*l* saliva.

Table 3 The odds ratios for the dental caries

(A)

	Crude odds ratio	95% CI	*P*-value	Adjusted odds ratio	95% CI	*P*-value
Salivary flow rate	0.753	0.605–0.936	0.011	0.866	0.677–1.107	0.249
Salivary pH	0.607	0.250–1.477	0.272			
Lactobacillus (log_{10} count)	1.643	1.449–1.864	<0.001	1.405	1.222–1.617	<0.001
Total Streptococci (log_{10} count)	1.835	1.334–2.523	<0.001	1.156	0.806–1.658	0.431
mutans streptococci (log_{10} count)	1.406	1.297–1.525	<0.001	1.246	1.135–1.368	<0.001
Dentifrice containing fluoride	0.837	0.585–1.196	0.328			
Fluoride varnish	1.343	0.570–3.164	0.500			
Fluoride mouth rinse	0.500	0.205–1.219	0.127			
Juice intake	1.409	1.107–1.794	0.005	1.236	0.950–1.607	0.114
Sweet snack intake	1.106	0.839–1.459	0.475			

(B)

	Crude odds ratio	95% CI	*P*-value	Adjusted odds ratio	95% CI	*P*-value
Salivary flow rate	0.640	0.410–0.999	0.049	0.651	0.402–1.054	0.081
Salivary pH	0.323	0.086–1.206	0.093			
Lactobacillus (log_{10} count)	1.566	1.290–1.900	<0.001	1.342	1.082–1.663	0.007
Total Streptococci (log_{10} count)	1.821	1.158–2.864	0.009	1.197	0.723–1.981	0.485
mutans streptococci (log_{10} count)	1.771	1.354–2.315	<0.001	1.378	1.029–1.874	0.032
Dentifrice containing fluoride	0.879	0.506–1.527	0.647			
Fluoride varnish	1.031	0.666–1.595	0.892			
Fluoride mouth rinse	0.521	0.215–1.256	0.150			
Juice intake	1.247	0.915–1.700	0.163			
Sweet snack intake	1.251	0.830–1.884	0.284			

Crude and multivariate adjusted odds ratios for subjects with df teeth or not (A) and for the high risks (B). Odds ratios were calculated by Logistic regression analysis. *P*-values were calculated by the Wald test. Among the 10 factors investigated in this study, only two factors such as salivary levels of mutans streptococci and Lactobacillus had statistically significant correlation with the dental caries conditions.

Table 4 Dose-response relationships of the odds ratios

(A)

		with or without dental caries			high risks		
	n	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
0	280	reference group			reference group		
$0<\log_{10}$ CFU/m$l<10^4$	103	3.112	1.809–5.355	<0.001	1.938	1.003–3.746	0.049
$10^4<\log_{10}$ CFU/m$l<10^5$	141	4.057	2.429–6.774	<0.001	4.331	2.515–7.457	<0.001
$10^5<\log_{10}$ CFU/ml	121	6.812	3.587–12.938	<0.001	6.327	3.665–10.992	<0.001

(B)

		with or without dental caries			high risks		
	n	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
0	133	reference group			reference group		
$0<\log_{10}$ CFU/m$l<10^5$	170	2.824	1.753–4.549	<0.001	1.139	0.552–2.315	0.725
$10^5<\log_{10}$ CFU/m$l<10^6$	180	3.651	2.248–5.929	<0.001	2.063	1.060–4.015	0.033
$10^6<\log_{10}$ CFU/ml	162	11.746	6.162–22.390	<0.001	5.649	2.981–10.706	<0.001

Dose-response relationships of the salivary levels of Lactobacillus (A) and mutans streptococci (B) for dental caries. Clear dose-response relationships were observed in this study.

Table 5 Cross table of the number of df teeth by the salivary levels of mutans streptococci and Lactobacillus

			mutans streptococci				
			0	$0<\log_{10}$CFU/m$l<10^5$	$10^5<\log_{10}$CFU/m$l<10^6$	$10^6<\log_{10}$CFU/ml	Total
Lactobacillus	0	mean ± SD	1.943 ± 2.917	2.471 ± 2.917	3.303 ± 3.371	4.048 ± 3.498	2.586 ± 3.148
		n	107	85	67	21	280
	$0<\log_{10}$CFU/m$l<10^4$	mean ± SD	3.250 ± 2.990	3.447 ± 3.244	4.108 ± 3.478	7.077 ± 5.327	4.147 ± 3.820
		n	8	45	37	13	103
	$10^4<\log_{10}$CFU/m$l<10^5$	mean ± SD	3.636 ± 4.501	4.516 ± 4.007	4.578 ± 3.621	7.250 ± 3.832	5.434 ± 4.058
		n	11	32	47	51	141
	$10^5<\log_{10}$CFU/ml	mean ± SD	2.857 ± 2.642	6.000 ± 3.640	5.103 ± 3.880	7.080 ± 4.341	6.277 ± 4.276
		n	7	8	29	75	121
	Total	mean ± SD	2.212 ± 3.070	3.280 ± 3.410	4.096 ± 3.610	6.726 ± 4.312	4.139 ± 3.989
		n	133	170	180	162	645

Dose-response relationship was observed by the mutans streptococci and Lactobacillus for df teeth. By two-way ANOVA analysis, *P*-values for the mutans streptococci was <0.001 and Lactobacillus <0.001. However, *P*-values for the mutans streptococci* Lactobacillus was 0.769, so interaction of the mutans streptococci and Lactobacillus for the number of df teeth was not observed in this study.

from 20.6% of the children, and salivary levels of Lactobacillus were not detected from 43.4% of the children (Fig. 2). However, more than 10^6 CFU/ml salivary mutans streptococci were detected from 25.1% of children.

To correlate the prevalence of the number of df teeth and risk factors investigated in this study, we performed Logistic regression analysis to calculate the odds ratio for df teeth. Table 3 shows the result of the crude odds ratios. Among 10 factors investigated in this study, salivary flow, amount of Lactobacillus, amount of total Streptococci, amount of mutans streptococci and daily number of sweet soft drinks taken were correlated with the prevalence

of dental caries. To eliminate the confounding factors, we performed multivariate Logistic regression analysis using the six factors that correlated with the dental caries. Table 3 shows the results of multivariate Logistic regression analysis. Only two of the five factors, amounts of mutans streptococci and Lactobacillus, were correlated with the prevalence of dental caries. For the medians of the decayed teeth, multivariate adjusted odds ratio were 1.259 by Lactobacillus and 1.105 by mutans streptococci; for the medians of the decayed or filled teeth, were 1.236 by Lactobacillus and 1.160 by mutans streptococci. The same tendencies were observed in the crude odds ratio if the children were divided into groups with or without decayed teeth or divided into two groups by the 75th percentile of the decayed or filled teeth (Table 3-B). To confirm the dose-response relationships, we categorized these factors by the distribution and then performed Logistic analysis again. Table 4 shows the dose-response relationships for these factors. Clear dose-response relationships were observed for the salivary levels of mutans streptococci and Lactobacillus. Then to check the co-effect of the mutans streptococci and Lactobacillus for dental caries, two-way ANOVA analysis was carried out. As shown in Table 5, no co-effects were found in mutans streptococci and Lactobacillus for dental caries.

Discussion

The etiology of dental caries has been suggested to be classified into three main categories, and this has been confirmed by laboratory investigation and clinical studies. In particular, mutans streptococci and Lactobacillus have been intensively studied as microflora factors. The nature of the acid production and biofilm formation by cariogenic microorganism has been studied[13,14]. However, clinical studies have shown that some subjects have decayed teeth even though these bacteria were below detection levels in the oral cavity[15,16]. These results have shown that the etiology of the dental caries cannot be explained easily. In contrast, some studies have shown that, as a substrate factor, dietary intervention to restrict the sucrose consumption has reduced the prevalence or incidence of the dental caries[6]. However, in this study, no correlation was found in daily sucrose consumption and the prevalence of the decayed teeth, filled teeth or df teeth. This may be because the method of surveying sucrose consumption by questionnaire does not reflect the actual conditions for the substitute factors. However, in present, there is no other method to survey for these factors.

The difference in the results may thus be due to the method of surveillance.

This study found no correlation in fluoride usage by dentifrice and mouth rinse and varnish. Clearly, fluoride has been used to reduce the prevalence of dental caries. In this study, the institutions that applied the fluoride were varied. This may also be due to the methods of the investigation. The method of the fluoride application and intervals of application may be reflected in the result.

Some studies have shown that salivary flow rate and salivary buffering capacity or salivary pH contribute to the incidence of dental caries[17,18], while others found no correlation between these factors and dental caries[16,19]. In this study, we could not find any statistically significant correlation between dental caries prevalence and salivary factors. It is generally considered that salivary flow rate was affected by the side effects of medication or systemic diseases. In general, the salivary flow rate of children is high, and few children take drugs affecting the sympathetic nerve system and reducing the salivary flow rate. Sgan-Cohen *et al.* found a significant correlation of the salivary flow and dental caries, however the correlation was weaker for other factors such as microflora[21]. This may be the main reason for the contradictory result.

In conclusion, of the three main factors suggested by Keyes, the microflora factors are strongly correlated with the prevalence of dental caries for the preschool children in Japan. In the future, controlling this factor for the cohort may lead to strong strategies for preventing dental caries through community-based prevention programs.

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Controlling cariogenic bacteria by the regular check-up system

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Reprinted from

PEDIATRIC DENTAL JOURNAL 14(1): 95–101, 2004

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Controlling cariogenic bacteria by the regular check-up system

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Abstract Regular check-ups are important for reducing the risk factors of dental caries. Through regular check-ups, clinicians empirically know that the incidence of the new dental caries was suppressed. However, the effects of the regular check-up system have not been thoroughly evaluated. Our primary concern was to evaluate the efficacy of the regular check-up system with professional preventive care for preventing dental caries. In this study, we evaluated attitudes toward regular check-ups. Five hundred and thirteen patients who visited one dental office in Japan from 1981 to 2000 and who were under 12 on the first visit were examined for dental caries, salivary mutans streptococci, and Lactobacilli to obtain baseline values and the values for the more recent visit analyzed in this study. Salivary mutans streptococci and Lactobacilli were counted using Dentocult SM and Dentocult LB. Most of the risk factors, particularly the salivary levels of the mutans streptococci, were reduced by regular check-ups in this study. There was a greater risk reduction in particular for the salivary levels of mutans streptococci in patients undertaking regular check-ups. Reduced salivary levels of Lactobacilli were also observed. However, the changes between the groups in the attitude toward regular check-ups were not statistically significant. This result indicates that most of the risk factors investigated in this study could be reduced by regular check-ups, particularly the levels of mutans streptococci, which has been suggested to be a strong etiology of dental caries.

Key words
Lactobacilli,
Mutans streptococci,
Regular check-up system,
Risk factors

Introduction

It has been suggested that regular check-ups for dental caries effectively reduce the incidence of dental caries[1,2]. The current national guidelines for preventing dental caries emphasize the importance of regular check-ups[3,4]. However, compliance and attitude toward preventing dental caries are not evaluated at these check-ups.

Received on September 29, 2003
Accepted on January 14, 2004

The risk factors of dental caries have been classified into three main categories—teeth, substrate, and oral micro flora[5]. Characteristics of micro flora, such as the oral levels of mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) and Lactobacilli, were suggested to be the most important risk factors[6,7]. These bacteria have been evaluated for the effects of the caries preventive programs. It has been suggested that salivary levels of the mutans streptococci can be reduced by using anti-microbial drugs[8]. However, in the conventional regular check-ups and treatment for preventing

dental caries, professional tooth cleaning and application of fluoride have sometimes been used and sometimes not used[9-11]. These strategies suppressed the incidence of dental caries. However, whether or not major risk factors such as salivary levels of these bacteria could be reduced was uncertain.

This study investigates the efficacy for the prevention of dental caries by regular check-ups with the professional preventive care and effect of the compliance with regular check-ups. It also investigates whether or not the salivary levels of the mutans streptococci and Lactobacilli could be reduced by the regular check-ups without using anti-microbial drugs.

Materials and methods

Subjects and evaluation method

The caries risks were evaluated for all the patients at one of the private dental office in Japan that participated, using the methods described by Petersson *et al.*[12] Two thousand one hundred and thirty-two patients under the age of 20 visit the private dental clinic for professional preventive programs since 1980 when this dental office opened. At 1981, this private dental office began to construct the database for the management of the patient's data, especially for the oral conditions and risks for the dental caries and periodontal disease.

Of these patients, 448 patients who visited the dental office from 1981 to 2000 and who were under 12 years of age at the first visit were examined for dental caries and salivary mutans streptococci and Lactobacilli to obtain baseline values and the values for the most recent visit analyzed in this study. Before the examination, informed consent was obtained to use the data of the oral conditions and the results of the saliva test for the construction of the data base and the possibility to use the data for publication. The examination of the dental caries was carried out by the dental hygienist, then checked again by the dentist and both dental hygienist and dentist had the clinical experience more than 10 years.

The Salivary mutans streptococci and Lactobacilli were counted using commercially available mutans streptococci and Lactobacilli evaluation kits, Dentocult SM and Dentocult LB (Orion Diagnostica Co. Ltd., Epsom, Finland). The results of this test were categorized according to the manufacturer's instructions.

Regular check-ups and preventive treatment

During a regular check-ups, dental plaque, one of the risk factors for dental caries[9,10], is controlled by the dentist or dental hygienist by professional tooth cleaning. Fluoride is then applied by the dentist or dental hygienist[6]. The risks of micro flora are reduced through this intervention to control dental plaque.

In a professional care program, the dentist or dental hygienist cleans the tooth surfaces by hand brushing and dental floss. 2% APF-containing paste (Fluorident gel, Stone Pharmaceuticals, Philadelphia, USA) is applied to the tooth surface by the toothbrush then indicated for the patients to bite a cotton roll for 5 min. Then, patients rinsed out one or twice and prohibited not to rinse for 30 min.

Fissure sealant or scaling to remove the dental calculus is performed if necessary. In addition, instructions regarding diet and using the fluoride containing toothpaste (950 ppm) are provided.

Statistical analysis

Prior to the analysis, the patients were classified into groups of regular attendees, irregular attendees, and those who never attended regularly, according to their compliance with the regular check-ups. The criteria were designated as follows. Regular attendees were present for regular check-ups every three months after the caries treatment was finished, irregular attendees included patients who understood the importance of regular check-ups but who occasionally missed their regular check-ups. The remainders were patients who visited the dental office only when they were experiencing dental problems. These patients were advised to attend the dental office regularly; however they were never attended without experiencing dental problems. The data of these patients used in this study as check-ups were the data at the attended dental office when experiencing dental problems. The number of patients who developed new dental caries during the check-up periods and the mean number of incidences of new dental caries were calculated for each group. The baseline characteristics of these groups were checked by the one-way ANOVA. Logistic regression analysis was then used to evaluate the attitude for the regular check-ups, to calculate the crude odds ratios and the adjusted odds ratios associated 95% confidence intervals. The results were adjusted by factors that had co-relation

Table 1 Incidence of the new dental caries in this study

		Percentage of subjects with new dental caries	*P*-value	Number of new dental caries (mean ± SD)	*P*-value
	n	% (n)		Number of teeth	
Total	448	30.4% (136)	0.006	0.76 ± 1.54	<0.001
Regular check-ups	273	25.6% (70)		0.47 ± 0.98	
Irregular check-ups	72	30.6% (22)		0.86 ± 1.51	
No check-ups	103	42.7% (44)		1.45 ± 2.34	

Percentage of subjects with new dental caries and the mean number of new dental caries classified by their compliance with regular check-ups. *P*-values were calculated by the Chi-square test for the percentage of subjects with new dental caries and two-way ANOVA for the mean number of the new dental caries.

Table 2 Baseline characteristics of the subjects participating in this study

(A)

	Regular check-ups	Irregular check-ups	No check-ups	Total	*P*-value
n	271	69	97	437	
Mean age at first visit	8.82 ± 2.84	10.47 ± 3.75	11.36 ± 3.78	9.67 ± 3.39	<0.001
Mean treatment periods	1.19 ± 1.45	1.24 ± 1.23	1.25 ± 1.42	1.21 ± 1.42	0.931
Mean follow up periods	3.61 ± 1.98	3.03 ± 1.84	2.43 ± 1.96	3.27 ± 2.01	<0.001
dft at baseline	0.74 ± 1.74	2.08 ± 3.21	3.27 ± 4.22	1.55 ± 2.95	<0.001

(B)

	Crude odds ratio	95% CI	*P*-value	Adjusted odds ratio	95% CI	*P*-value
Age at first visit	1.056	0.993–1.123	0.084			
Follow-up periods	1.061	0.973–1.157	0.179			
dft at baseline	1.090	1.029–1.115	0.004			
Irregular check-ups	1.091	0.624–1.909	0.760	1.147	0.586–2.246	0.689
No check-ups	2.250	1.382–3.662	0.001	2.358	1.241–4.482	0.009

(A) shows the baseline characteristics for each group for the attitude of regular check-ups. *P*-values were calculated by one-way ANOVA. Statistically significant differences were observed in each group except for the treatment periods.
(B) shows the results of the crude and adjusted odds ratios from logistic regression analysis.

with the baseline characteristics in each group.

The relative risk reduction (RRR) and absolute risk reduction (ARR) were calculated. The numbers needed to treat (NNT) for the regular check-ups was then calculated using the inverse of the absolute risk reduction.

To determine the attitude for the regular check-ups and to evaluate whether salivary cariogenic bacteria were reduced or not, the methods of Friedman were used to check the difference of the salivary levels of the mutans streptococci and Lactobacilli in each group. *P*-values less than 0.05 were considered statistically significant.

Results

Sixty-five (12.9%) of the 513 patients dropped out. The main reasons were as follows: relocation 29 subjects (44.6%), and cancelled the check-ups and never come to the dental office 25 subjects (38.5%). The demographics of the patients who participated in this study were as follows. There were 297 males (45.5%) and 356 females (54.5%), the mean age at the first visit was 5.77 ± 3.10, the distribution was less than 5 years old: 224 (50%), 5–10 years old: 182 (40.6%), 11 or 12 years old 42 (9.4%) and the mean follow-up period was 4.22 ± 2.25 years.

Table 1 shows the percentage of subjects with

Table 3 NNT for the regular check-ups

	RRR	ARR	NNT
Regular check-ups vs. No check-ups	40.0	17.1	5.9
Regular check-ups vs. Irregular check-ups	16.1	12.2	8.2

Relative risk reduction, absolute risk reduction and numbers needed to treat (NNT) for the attitude of the regular check-ups. NNT was calculated by the inverse of the absolute risk reduction.

Table 4 Changes in the salivary levels of the mutans streptococci evaluated by Dentocult SM

(A)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	30	11.0	39	14.3	74	27.1	<0.001
1	41	15.0	45	16.5	51	18.7	
2	78	28.6	81	29.7	77	28.2	
3	124	45.4	108	39.6	71	26.0	

(B)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	6	8.2	5	6.5	6	7.8	0.399
1	12	16.5	14	19.4	17	23.4	
2	26	36.4	30	41.9	29	40.6	
3	28	38.9	23	32.3	20	28.1	

(C)

	First visit		Treatment finished		Check-ups		
Level	n	%	n	%	n	%	*P*-value
0	8	7.8	16	15.3	17	16.0	0.038
1	16	15.6	12	11.5	17	16.0	
2	37	35.6	42	40.3	40	38.7	
3	42	41.1	34	32.7	30	29.1	

Table 4 shows the results of number and percent of subjects for the changes in salivary levels of the mutans streptococci on each visit. (A) indicates the regular attendees, (B) irregular attendees, and (C) no check-ups. Data were analyzed by Friedman Test. A statistically significant reduction of the salivary levels of mutans streptococci was observed in regular check-up patients.

new dental caries and the mean number of new dental caries, classified by their attitude toward regular check-ups. The result clearly illustrates that regular check-ups reduce the incidence of new dental caries. This result was found statistically significant by one-way ANOVA.

Baseline characteristics of the patients in each group are shown in Table 2-A. Statistically significant differences were found in mean age of the first visit, mean follow-up periods and baseline dft between each group. Patients were divided into two groups depending on whether they had new dental caries or not, and only the baseline DMFT was correlated with the incidence of dental caries (data not shown).

We performed logistic regression analysis to investigate the odds ratios of the attitude towards