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Effect of mixed mutans streptococci colonization on caries development

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Objective: To evaluate the clinical importance of mixed mutans streptococci colonization in predicting caries in preschool children.
Methods: Caries prevalence was examined twice, with a 6-month interval, in 410 preschool children aged 3–4 years at baseline. A commercial strip method was used to evaluate the mutans streptococci score in plaque collected from eight selected interdental spaces and in saliva. Mutans streptococci typing polymerase chain reaction (PCR) assays (*Streptococcus sobrinus* and *Streptococcus mutans*, including serotypes c, e, and f) were performed using colonies on the strips as template.
Results: Twenty variables were examined in a univariate analysis to predict caries development: questionnaire variables, results of clinical examination, mutans streptococci scores, and PCR detection of *S. sobrinus* and *S. mutans* (including serotypes c, e, and f). Sixteen variables showed statistically significant associations ($P < 0.04$) in the univariate analysis. However, when entered into a logistic regression, only five variables remained significant ($P < 0.05$): caries experience at baseline; mixed colonization of *S. sobrinus* and *S. mutans* including *S. mutans* serotypes; high plaque mutans streptococci score; habitual use of sweet drinks; and nonuse of fluoride toothpaste.
Conclusion: 'Mixed mutans streptococci colonization' is a novel measure correlated with caries development in their primary dentition.

Key words: dental caries; mutans streptococci; preschool children; serotype

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Mutans streptococci are the main etiologic agents of dental caries in humans (11, 15) and are classified into seven species (15, 28). Of these, *Streptococcus sobrinus* and *Streptococcus mutans* are the most frequently isolated from the human oral cavity (11, 15). Moreover, research indicates that the coexistence of *S. sobrinus* and *S. mutans* is an important factor in the development of dental caries (2, 13, 14). In 2000, oligonucleotide primers specific for portions of their glucosyltransferase genes (*gtfB* of *S. mutans* and *gtfI* of *S. sobrinus*) were designed and these should prove useful in epidemiologic studies to evaluate the prevalence of these organisms (18).

S. mutans strains are classified into three serotypes (c, e, and f), and the serologic specificity is defined by rhamnose-glucose polysaccharide on the cell wall (15). Recently, the genes required for glucose side chain formation of the serotype c-specific rhamnose-glucose polysaccharide were identified and characterized (21), and we elucidated the loci responsible for determining the other serotypes (26). In that study (26), we developed three pairs of primers that were specific for each serotype, and a multiplex PCR assay readily identified serotypes of *S. mutans* strains. Using the same cross-sectional samples in this study, the relationship was identified between the results of the *S. mutans* serotyping PCR assays and caries experience in preschool children. Clearly, a caries development study should be done to identify the clinical usefulness of the assay.

Furthermore, it is reasonable to identify caries-susceptible individuals from the correlation between the presence of mutans streptococci and caries development (3, 6, 31). Using the aforementioned mutans streptococci typing PCR assays (*S. sobrinus* and *S. mutans*, including serotypes c, e, and f), it might be possible to identify caries-susceptible individuals more clearly. In this study, in order to evaluate the clinical importance of the mutans streptococci typing PCR assay in

predicting caries development, we investigated the multifactorial etiology of caries development in 410 children 6 months after we performed baseline examinations on them.

Material and methods

Subjects

This study examined 432 children (237 males and 195 females) aged 3–4 years at baseline, who attended five preschools in Tokyo, Japan, which is a nonfluoridated area. The ethics committee of Nihon University School of Dentistry approved this study. The parents of all of the children were asked to complete a questionnaire and to allow us to examine their children's teeth and to take microbiologic samples. Six months after the baseline measurements, 416 children were re-examined. Sixteen children from the baseline study were lost because they left the schools or did not attend the examination. Six children were eliminated from the analysis for the reason described below. Data from 410 children (mean age = 4.2 - years at baseline; 225 males and 185 females) were analyzed in the 6-month follow-up study.

Clinical examination

Caries prevalence was examined by two calibrated examiners (Cohen's kappa = 0.88) on two occasions, 6 months apart, between November 2001 and May 2002. Caries per tooth surface were diagnosed visually without radiographs (29). Only manifest lesions accompanied by enamel loss or cavities involving the dentin of the primary teeth were considered.

The labial, buccal, and lingual tooth surfaces were inspected visually for the presence of dental plaque, without using disclosing solution. Children with visible plaque on one or more tooth surfaces were categorized as positive (visible plaque).

Bacteriologic examination

At baseline, the Dentocult SM® Strip mutans and Site Strip (Orion Diagnostica, Espoo, Finland) were used to evaluate mutans streptococci levels in unstimulated saliva and plaque. To sample saliva, the strip was rotated on the surface of the tongue 10 times. To sample plaque, site-specific plaque samples were collected from eight specified interdental spaces using a wooden toothpick: 54/55, 52/53, 51/61, 62/63, 64/65, 74/75, 71/81, and 84/85, as previously described (23). Each Dentocult SM® kit contains two pairs of plastic test strips, each of which has four separate pads. One plaque sample was placed on one pad, and two strips were incubated per broth vial, as previously described (23). After incubation for 48 h at 37°C, the strips for saliva and plaque were dried, examined using a microscope at 10× magnification, and scored from 0 to 3 according to the manufacturer's instructions. One dentist experienced in this method determined all the scores. The highest plaque score of eight tested regions for each subject was used as the individual plaque mutans streptococci score as described previously (25). Colonies on the two strips for plaque were used for PCR detection as described previously (26).

PCR experiments

Rapidly isolated chromosomal DNA from whole colonies on the Dentocult SM® strips was used as template. PCR experiments designed to discriminate between *S. mutans* and *S. sobrinus* targeted the gene encoding the water-insoluble glucan-synthesizing enzyme (GTF-I), by using two sets of primers (GTFB-F plus GTFB-R and GTFI-F plus GTFI-R), as described previously (18). Three sets of primers (SC-F plus SC-R, SE-F plus SE-R, and SF-F plus SF-R) were used in the PCR assay to identify *S. mutans* serotypes (c, e, and f), as described previously (26). The primers used in this study are shown in Table 1.

Statistical analysis

Data were analyzed using the SPSS® software package (SPSS, Chicago, IL) and the statistical program EXCEL Toukei (Esumi, Tokyo, Japan). The differences in the number of tooth surfaces with caries increment over a half-year period (Δdfs) between groups were examined using the Kruskal–Wallis test followed by the Steel–Dwass multiple comparison test. Differences in caries development between groups were tested using the Chi-squared test or Fisher's exact test. Single and multiple logistic regression analyses were used to define the predictors of caries development.

Results

Epidemiologic data

New caries lesions were detected in 170 children on the second visit. The caries prevalence in the 410 children increased from 43.2% at baseline to 51.2% at follow-up. The mean ± SE dfs in the subjects was 3.45 ± 0.32 (ds, 1.72 ± 0.22; fs, 1.73 ± 0.19) at baseline and 5.08 ± 0.41 (ds, 2.47 ± 0.25; fs, 2.61 ± 0.25) at follow-up.

Bacteriologic data and its relationship with clinical findings

The distribution of the mutans streptococci score was 53%, 7%, 13%, and 27% in plaque, and 65%, 17%, 10%, and 8% in saliva for scores 0, 1, 2, and 3, respectively. Sixty-one percent of the children had identical plaque and saliva mutans streptococci scores.

The subjects were divided into two groups based on the most valid criteria in screening caries development for the plaque (between scores 1 and 2) and saliva mutans streptococci scores (between scores 0 and 1), respectively. The subjects were also split into groups according to whether caries experience was present at baseline. The relationship between caries development and mutans streptococci scores/caries prevalence at baseline was ascertained (Table 2). There was a significant correlation between the plaque mutans streptococci score and caries development, regardless of caries prevalence at baseline (Table 2a; χ^2 test, $P < 0.001$). There was a significant relationship between caries development and saliva mutans streptococci score when

Table 1. PCR primers used in this study

Species	Primer	Sequence (5′ to 3′)	(Ref.)
S. mutans	GTFB-F	ACTACACTTTCGGGTGGCTTGG	(18)
	GTFB-R	CAGTATAAGCGCCAGTTTCATC	
S. sobrinus	GTFI-F	GATAACTACCTGACAGCTGACT	(18)
	GTFI-R	AAGCTGCCTTAAGGTAATCACT	
S. mutans	SC-F	CGGAGTGCTTTTTACAAGTGCTGG	(26)
serotype c	SC-R	AACCACGGCCAGCAAACCCTTTAT	
S. mutans	SE-F	CCTGCTTTTCAAGTACCTTTCGCC	(26)
serotype e	SE-R	CTGCTTGCCAAGCCCTACTAGAAA	
S. mutans	SF-F	CCCACAATTGGCTTCAAGAGGAGA	(26)
serotype f	SF-R	TGCGAAACCATAAGCATAGCGAGG	

Table 2. Relationship of plaque mutans streptococci and salivary mutans streptococci scores and caries prevalence at baseline to caries development

Caries prevalence at baseline	Plaque mutans streptococci score			
	Low (0 or 1)	High (2 or 3)	Total	*P*-value§
Without caries	13 (187) *	35 (46)	18 (233)	<0.001
With caries	57 (60)	81 (117)	73 (177)	<0.001
Subtotal	24 (247)	68 (163)	41 (410)	<0.001
	Salivary mutans streptococci score			
	Low (0)	High (1, 2 or 3)	Total	*P*-value†
Without caries	15 (195) *	32 (38)	18 (233)	0.013
With caries	65 (72)	78 (105)	73 (177)	0.060
Subtotal	28 (267)	66 (143)	41 (410)	<0.001

*Percentage of subjects with caries development (number of subjects).
§Difference according to the plaque mutans streptococci score: statistical evaluation using the χ^2 test.
†Difference according to the salivary mutans streptococci score: statistical evaluation using the χ^2 test.

caries experience was absent at baseline (Table 2b; χ^2 test, $P = 0.013$). However, there was no significant difference in caries development between the high and low saliva mutans streptococci score groups when caries experience was present at baseline (Table 2b; χ^2 test, $P = 0.060$).

Discrimination of *S. sobrinus* and *S. mutans* colonization, including *S. mutans* serotypes, and its relationship with clinical findings

Mutans streptococci was isolated from 208 of 416 children using the Dentocult SM kit, and two kinds of PCR detection were done. The PCR methods used did not amplify a band in only six of 208 children from whom mutans streptococci was isolated using the Dentocult SM kit. Consequently, these six children were eliminated from the subsequent statistical analyses. The prevalence of *S. sobrinus* and *S. mutans*, including *S. mutans* serotypes, is represented in Table 3.

The mean ± SE Δdfs for each group of children with different types of colonization with mutans streptococci was 0.77 ± 0.19 in the 'score 0' group; 0.71 ± 1.04 in the 'colonization with *S. sobrinus* only' group; 2.39 ± 0.22 in the 'colonization with one *S. mutans* serotype' group; 5.91 ± 0.83 in the 'mixed colonization with multiple *S. mutans* serotypes' group; and 4.03 ± 0.51 in the 'mixed colonization with *S. sobrinus* and *S. mutans'* group. The differences in Δdfs among the groups were statistically significant using the Kruskal–Wallis test ($P < 0.001$).

To clarify the effect of mixed colonization of mutans streptococci on caries increment, the two mixed colonization groups ('mixed colonization with multiple *S. mutans* serotypes' and 'mixed colonization with *S. sobrinus* and *S. mutans'* groups) were combined into a 'mixed colonization' group. The mean ± SE Δdfs for the 'mixed colonization' group was 4.55 ± 0.66 and the differences among the four groups were statistically significant with the Kruskal–Wallis test ($P < 0.001$). The results of the Steel–Dwass multiple comparison test indicated significant differences between the 'mixed colonization' group and the 'score 0' ($P < 0.01$); 'colonization with *S. sobrinus* only' ($P < 0.05$) and 'colonization with a single *S. mutans* serotype' groups ($P < 0.01$); and also between the 'score 0' and 'colonization with a single *S. mutans* serotype' groups ($P < 0.01$; Fig. 1).

Furthermore, the subjects were split into groups according to whether caries experience was present at baseline; the relationship of 'mixed colonization' and caries prevalence at baseline to caries development was determined and is shown in Table 4. 'Mixed colonization' was significantly correlated with caries development regardless of caries prevalence at baseline (χ^2 test, $P = 0.005$ without caries at baseline and $P = 0.032$ with caries at baseline).

Single and multiple logistic regression analyses

The dichotomized risk indicators were further analyzed using a logistic regression. A univariate analysis examined the ability of each of 20 variables to predict caries development. Sixteen variables showed statistically significant associations (Table 5). The following variables did not show a significant association with caries development: gender; habitual con-

Table 3. Prevalence of *S. sobrinus* and *S. mutans*, including *S. mutans* serotypes

Mutans streptococci	Group*	No. of subjects
Without mutans streptococci	A	208
S. sobrinus	B	7
S. mutans serotype c	C	138
S. mutans serotype e	C	16
S. mutans serotype f	C	1
S. mutans serotype c and e	D	7
S. mutans serotype c and f	D	3
S. mutans serotype c, e, and f	D	1
S. sobrinus and *S. mutans* serotype c	D	24
S. sobrinus, and *S. mutans* serotype c and e	D	2
S. sobrinus and *S. mutans* serotype e	D	3
Total		410

*A, subjects scoring 0 with the Dentocult SM kit ($n = 208$); B, subjects colonized with *S. sobrinus* only ($n = 7$); C, subjects colonized with a single serotype of *S. mutans* ($n = 155$); D, subjects with a mixed colonization of *S. sobrinus* and *S. mutans*, including *S. mutans* serotypes ($n = 40$).

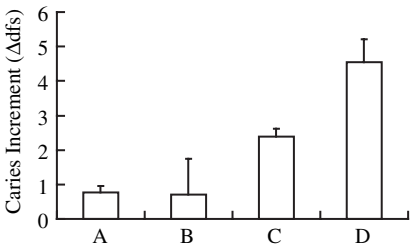

Fig. 1. Caries increment (Δdfs) over 6 months in groups of children with different types of colonization with mutans streptococci. A, subjects scoring 0 with the Dentocult SM® kit ($n = 208$); B, subjects colonized with *S. sobrinus* only ($n = 7$); C, subjects colonized with a single serotype of *S. mutans* ($n = 155$); D, subjects with a mixed colonization of *S. sobrinus* and *S. mutans*, including *S. mutans* serotypes ($n = 40$). The vertical bars represent the standard error. Differences in caries increment (Δdfs) among the groups were significant using the Kruskal–Wallis test ($P < 0.001$). There were significant differences between group D and each of groups A ($P < 0.01$), B ($P < 0.05$), and C ($P < 0.01$), and between groups A and C ($P < 0.01$) using the Steel–Dwass multiple comparison test.

Table 4. Relationship of mixed colonization and caries prevalence at baseline to caries development

Caries prevalence at baseline	Mixed colonization			
	Absent	Present	Total	*P*-value§
Without caries	16 (226)*	57 (7)	18 (233)	0.005†
With caries	69 (144)	88 (33)	73 (177)	0.032
Subtotal	37 (370)	83 (40)	41 (410)	< 0.001

*Percentage of subjects with caries development (number of subjects).
§Difference according to the mixed colonization: statistical evaluation using the χ^2 test.
†This result was also confirmed by Fisher's test ($P = 0.020$).

Table 5. Single risk indicators for predicting caries development in 3–4-year-olds. Logistic regression analysis for single variables

Variables observed at baseline	% of positive children	OR	95% CI for OR	*P*-value
Caries prevalence at baseline	43	12.59	7.92–20.42	< 0.001
Visible plaque	74	2.49	1.55–4.10	< 0.001
Habitual use of sweet drinks	12	2.93	1.58–5.59	< 0.001
Brushing frequency < 3 times/day	89	2.30	1.16–4.91	0.022
Fluoride toothpaste nonuse	39	1.60	1.07–2.39	0.023
Parents clean teeth	23	1.66	1.04–2.64	0.032
Frequent snacks	42	1.63	1.09–2.43	0.017
Plaque mutans streptococci score > 1	40	6.80	4.41–10.65	< 0.001
Salivary mutans streptococci score > 0	35	4.82	3.13–7.50	< 0.001
S. mutans	48	5.28	3.46–8.16	< 0.001
S. sobrinus	9	4.85	2.30–11.19	< 0.001
S. mutans serotype c	43	4.94	3.25–7.59	< 0.001
S. mutans serotype e	7	2.89	1.34–6.64	0.009
Multiple serotypes of *S. mutans* only	3	6.65	1.69–44.00	0.016
S. mutans and *S. sobrinus*	7	7.73	3.12–23.34	< 0.001
Mixed colonization*	10	8.02	3.65–20.19	< 0.001

*Mixed colonization of *S. sobrinus* and *S. mutans*, including *S. mutans* serotypes.

sumption of sweet snacks; nonuse of sugar substitutes; and detection of serotype f. In the multivariate analysis, five variables (caries experience at baseline; mixed *S. sobrinus* and *S. mutans* colonization including *S. mutans* serotypes; high plaque mutans streptococci score (> 1); habitual use of sweet drinks; and nonuse of fluoride toothpaste) remained in the final model ($P < 0.05$; Table 6).

Discussion

At the final model in this study, there were five significant indicators:

- caries experience at baseline;
- high plaque mutans streptococci score (> 1);
- habitual use of sweet drinks;
- nonuse of fluoride toothpaste;
- mixed *S. sobrinus* and *S. mutans* colonization including *S. mutans* serotypes (Table 6).

The first three indicators concur with earlier findings regarding the association of these variables with caries development at preschool ages (9, 22).

Recently, using the same commercial kit and multivariate approach, we concluded that the plaque mutans streptococci score and past caries experience at baseline were the best predictors of caries development in Japanese preschool children (25). In that study, the evaluation of plaque mutans streptococci sampled from only two proximal sites on the molars resulted in valid outcomes. If the number of detection sites were increased, the outcome might be improved. However, collecting whole plaque from all tooth surfaces is difficult and unrealistic when clinical convenience is taken into consideration. The strip included in this kit has four sites onto which plaque is transferred. If an additional strip were provided in the kit, plaque from eight regions could easily be evaluated, as two strips can be incubated in the single broth vial provided. Although the manufacturer's instructions state that only one strip should be incubated per vial, we confirmed the validity of evaluating eight sites using two strips per vial (23). In this study, we used two strips and incubated them in the single broth vial provided. Subsequently, plaque from eight regions was evaluated, allowing us to estimate the plaque mutans streptococci level for a broader area. By using this eight-region method, there was an excellent potential for predicting an individual's caries development (Tables 5 and 6).

Table 6. The variables in the final model for predicting caries development in preschool children. Multiple forward stepwise logistic regression analysis

Risk indicator	B	SE	OR	95% CI	*P*-value
Caries prevalence at baseline	1.05	0.13	8.14	4.89–13.77	< 0.001
Mixed colonization*	0.54	0.25	2.96	1.14–8.58	0.033
Plaque mutans streptococci score > 1	0.51	0.13	2.75	1.62–4.67	< 0.001
Habitual use of sweet drinks	0.41	0.20	2.29	1.06–5.05	0.037
Nonuse of fluoride toothpaste	0.31	0.13	1.85	1.11–3.11	0.020
Constant	− 0.63	0.30			0.035

*Mixed colonization of *S. sobrinus* and *S. mutans*, including *S. mutans* serotypes.

Previous studies suggest that fluoride use is fundamentally important for decreasing the risk of caries (6). However, access to fluoride is limited in Japan, and as children are not given fluoridated water or fluoride tablets, fluoride toothpaste is the only available source of fluoride (17). In our previous study in 1995, only around 20% of preschoolers used fluoride dentifrices (24). For many years, caregivers in Japan were advised by dentists not to use dentifrices when brushing a child's teeth. Consequently, even today many caregivers do not use fluoride dentifrices. Interestingly, in this study, which investigated an area adjacent to that of the previous study (24) in Tokyo, Japan, between 2001 and 2002, the percentage of fluoride toothpaste users was 61% (Table 5), and this factor remained significant in the final model (Table 6). These results reconfirm the importance of using fluoride toothpaste to prevent caries in Japanese preschool children.

The caries prevalence at baseline (43.2%) in this study was similar to the results of the Japan National Oral Health Survey, which reported that the caries prevalence in Japanese children in 1999 was 41% in 4-year-olds (5).

The prevalence of *S. mutans* and *S. sobrinus* in children aged 3-4 years (mean age = 4.2 years) at baseline in this study was 48% and 9%, respectively: 41% were positive for *S. mutans* alone, 2% were positive for *S. sobrinus* alone, 7% were positive for both *S. mutans* and *S. sobrinus, and* 51% were negative for both *S. mutans* and *S. sobrinus* (Table 5). These rates are similar to those found in

previous studies that examined preschool children using a culture technique (7, 12). However, a recent study examining Japanese children reported that the prevalence of *S. mutans* and *S. sobrinus* in 3–5-year-olds ($n = 77$) was 73% and 61%, respectively: 25% were positive for *S. mutans* alone, 13% were positive for *S. sobrinus* alone, 48% were positive for both *S. mutans* and *S. sobrinus,* and 14% were negative for both *S. mutans* and *S. sobrinus* (19). Notably, the Okada study (19) found a higher level of *S. sobrinus* than we did in our results. Dissimilarities in study subjects and methodology may have contributed to the difference in findings. Unlike our preschool subjects, the Okada study subjects were visitors to a dental hospital. Moreover, the Okada study used a direct PCR detection technique from plaque samples.

Of the mutans streptococci serotypes, *S. mutans* serotype c strains predominate in the human oral cavity (10), which we confirmed: the percentages of *S. mutans* serotype c, e, and f, and *S. sobrinus* were 43%, 7%, 1%, and 9%, respectively. In a preliminary study, we compared the caries experience at baseline (dfs) using the same subjects in this study, but excluding children with *S. sobrinus* colonization, in three groups, subjects scoring 0 with the Dentocult SM kit; subjects with a mono colonization by a single serotype of *S. mutans*, *and* subjects with a mixed colonization by multiple serotypes of *S. mutans*. We found that the dfs in the mixed colonization by *S. mutans* multiple serotypes group was significantly higher than the dfs in the other two groups (26). In the present study, each of the Δdfs in two mixed colonization groups – 'mixed colonization with multiple *S. mutans* serotypes' and 'mixed colonization with *S. sobrinus* and *S. mutans'* groups – was higher than that in the 'score 0' group, the '*S. sobrinus* colonization only' group, and the 'single colonization with a *S. mutans* serotype' group (Fig. 1).

S. sobrinus has a high cariogenic potential in experimental animals (20, 27). We observed several cases of caries-free children who had *S. mutans* or *S. sobrinus* alone ($n = 28$, data not shown). In the recent Okada study, 13 of 77 (16.9%) children were caries-free; however, 7 (53.8%) of those had either *S. mutans* or *S. sobrinus* alone (19). Many studies have reported cases of children harboring both *S. mutans* and *S. sobrinus* who had a significantly higher caries incidence than those with *S. mutans* or *S. sobrinus* alone (2, 13, 14, 19, 30).

Research has shown that children with nursing-bottle dental caries were often colonized with more than one clonal type (1). Moreover, research suggests that *S. sobrinus* did not seem to play a major role in children aged 1–2½ years with rampant caries (16). 'Mixed colonization with multiple *S. mutans* serotypes' might be related to the occurrence of caries, especially in the youngest children. The results of these previous studies and of our present study suggest a close relationship between mixed mutans streptococci colonization and caries development.

Through the aforementioned considerations, we hypothesize that mixed colonization is an important factor in the development of caries. To clarify the effect of mixed colonization of mutans streptococci on caries increment, the two mixed colonization groups ('mixed colonization with multiple *S. mutans* serotypes' and 'mixed colonization with *S. sobrinus* and *S. mutans*') were combined into a 'mixed colonization' group. The Δdfs for the 'mixed colonization' group was significantly higher than for the other colonization groups (Fig. 1). Furthermore, in our study, the plaque mutans streptococci score and mixed colonization were valid regardless of caries experience at baseline (plaque mutans streptococci score, $P < 0.001$: mixed colonization without caries at baseline, $P = 0.005$; mixed colonization with caries at baseline, $P = 0.032$; Tables 2 and 4). Furthermore, in the single logistic regression analysis, 'mixed colonization' had the second highest odds ratio for predicting caries development of the 16 significant variables, following 'caries prevalence at baseline' (Table 5). In the multivariate analysis, this unique variable remained in the final model, and had the second highest odds ratio among the five significant indicators (Table 6).

Although *S. sobrinus* corresponds to mutans streptococci serotypes d and g, we do not at present have any PCR techniques that can distinguish between *S. sobrinus* serotypes. Therefore, it is possible that all seven subjects belonging to the '*S. sobrinus* colonization only' group were colonized by serotypes d and g. If this was the case, those seven children should be included in the 'mixed colonization' group. Nevertheless, even if all of the children included in the '*S. sobrinus* colonization only' group are actually in the 'mixed colonization' group, the Δdfs for the 'mixed colonization' group is still significantly higher than that for the other colonization groups (Steel–Dwass multiple comparison test, $P < 0.05$; data not shown). Furthermore, the results of a multiple logistic regression analysis revealed that mixed colonization remains one of five significant indicators in the final model ($P < 0.1$; data not shown). These results indicate that the inability to perform *S. sobrinus* serotyping in our study does not weaken our hypothesis that the mixed colonization of mutans streptococci is related to cariogenicity.

We find it difficult to explain how 'mixed infection' increases the cariogenicity of these bacteria. The 'mixed infection' seemed to be related to dietary habits that include sugar consumption. However, there was no evidence for a close relationship between the 'mixed infection' and four variables regarding dietary habits in this study: habitual use of sweet drinks; frequent snacks; habitual consumption of sweet snacks; and nonuse of sugar substitutes (χ^2 test, $P > 0.1$; data not shown).

As a result of our research, we posit a significant speculation: competition exists among mutans streptococci serotypes for their natural habitat, i.e. dental plaque on the tooth surface. Dental plaque is a community of bacteria (4), and the 'total activity' of the plaque influences the development of dental caries. Nevertheless, gaps remain in our basic knowledge of the interaction of these bacteria. As previously described (8), acquiring this information will require more detailed microbiological investigations using knowledge from population biologists and ecologists.

In conclusion, we identified the novel caries indicator 'mixed mutans streptococci colonization'. The presence of 'mixed mutans streptococci colonization' is correlated with developing caries in the primary dentition.

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