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PCR detection of *Streptococcus mutans* and *S. sobrinus* in dental plaque samples from Japanese pre-school children

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***Streptococcus mutans* and *S. sobrinus* are associated with the development of dental caries. These bacteria were detected by PCR and then their presence was compared with the incidence of dental caries in 77 Japanese pre-school children. Plaque samples were collected from all erupted tooth sites in the subjects, aged 3–5 years old and each with primary dentition, with a sterile toothbrush. A dental examination was performed for dmft (decayed, missing, filled, total) with the WHO caries diagnostic criteria. In all subjects, the prevalence of *S. mutans* and *S. sobrinus* was 72.8% and 61.1%, respectively; 19 (24.7%) were positive for *S. mutans* alone, 10 (13.0%) were positive for *S. sobrinus* alone, 37 (48.1%) were positive for both *S. mutans* and *S. sobrinus*, and 11 (14.3%) were negative for both *S. mutans* and *S. sobrinus*. The dmft scores of children positive for both *S. mutans* and *S. sobrinus* were significantly higher than those positive for *S. mutans* alone. These results indicate that children harbouring both *S. mutans* and *S. sobrinus* have a significantly higher incidence of dental caries than those with *S. mutans* alone.**

Introduction

Mutans streptococci (*Streptococcus mutans* and *S. sobrinus*) are closely associated with the development of dental caries in man. These bacteria are the most common pathogens isolated from human dental plaque and their prevalence has been reported in epidemiological studies [1, 2]. Some researchers have found that *S. mutans* is more prevalent than *S. sobrinus* in dental plaque samples [3, 4], whereas several epidemiological studies have shown that the prevalence of *S. sobrinus* is more closely associated with high caries activity [5, 6].

The primary differentiation of these cariogenic species has generally been based on their colonial morphology on mitis-salivarius (MS) or MS-bacitracin agar [7–9]. However, this conventional method is sometimes inaccurate, as well as time-consuming and laborious. Moreover, it has been reported that MS-bacitracin inhibits the growth of *S. sobrinus* to a greater extent than that of *S. mutans* [10, 11]. Thus, it is of great importance to distinguish the presence of these two species separately in children for accurate prediction and effective prevention of dental caries.

Various methods have been used for the detection of putative pathogens, including direct microscopy, cultivation, enzyme tests, enzyme-linked immunosorbent assays and species-specific DNA probes. It is now widely accepted that PCR methodology provides a more sensitive means of detection of putative bacteria species, when compared with conventional culture techniques [12–14], as it is able to detect low numbers of bacterial species with a detection limit of as few as 25–100 cells [12, 14, 15], while being quick and relatively simple to perform. Moreover, a PCR assay has been found to be suitable for the specific detection and identification of human cariogenic bacteria, such as *S. mutans* and *S. sobrinus* [12, 14–16].

The purpose of this study was to detect *S. mutans* and *S. sobrinus* by PCR, and to compare their presence with the incidence of dental caries in pre-school children.

Materials and methods

Seventy-seven Japanese pre-school children, aged 3–5 years old and each with primary dentition, who were

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visitors to Hiroshima University Dental Hospital were enrolled. Consent for participation was obtained from at least one of their parents before the study according to the ethical guidelines of the Declaration of Helsinki (1975). The subjects received a dental examination by two well-trained paediatric dentists (YS and TD) while seated in a dental chair. The WHO caries diagnostic criteria were used for determining the dmft (decayed, missing, filled, total) index [17]. Those who had taken antibiotics within the previous 3 months or with systemic diseases were excluded. The agreement between the examining dentists was >90% for inter-examiner reproducibility for dmft criteria in a sample of 20 of the subjects.

Plaque sampling

Dental plaque was collected by brushing all erupted teeth with a sterile toothbrush for 1 min as described previously [18]. Plaque adhering to the toothbrush was removed by washing several times in a tube of sterile distilled water. The plaque samples were immediately transported to the laboratory and stored at −20°C, before extraction of genomic DNA.

PCR

S. mutans JCM5175^T and *S. sobrinus* ATCC27607^T were used as controls. PCR detection of tested species was performed with the primers described by Igarashi *et al*. [12, 15], while that of 16S ribosomal DNA was done by the method of Goncharoff *et al*. [19].

Plaque samples were first harvested by centrifugation at 1600 *g* for 20 min. The supernate were discarded and individual cell pellets were stored at −20°C until DNA isolation. The genomic DNA preparation from each plaque sample was obtained by a standard miniprep procedure [20], with the additon of an RNAase treatment [21]. DNA concentrations in the dental plaque samples were calculated by measuring A_{260}, and the quality was estimated by the A_{260}/A_{280} ratio [22].

PCR amplification was performed in a reaction mixture (25 μl) consisting of PCR beads (Amersham Pharmacia Biotech, AB, Uppsala, Sweden) that contained an enzyme and the required reagents, 25 pmol of each primer and 20−50 ng of the template DNA solution in a thermal cycler (Program Temp Control System PC-700, ASTEC, Fukuoka, Japan). Positive and negative controls were included in each PCR set and for all sample processing. The reaction mixture was denatured at 95°C for 3 min followed by a series of amplifications: denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The series was repeated for 26 cycles. The final cycle comprised 94°C for 1 min, 55°C for 1 min and 72°C for 5 min [12]. After amplification, 15 μl of the PCR products were analysed by electrophoresis on an agarose 1.2% gel. The newly synthesised DNA fragments were visualised under ultraviolet light at 302 nm after staining with ethidium bromide. The size of the PCR products was estimated from the electrophoretic migration of products relative to a 100-bp ladder (Amersham Pharmacia Biotech, AB, Uppsala, Sweden).

Statistical analysis

The Mann-Whitney U-test was employed for the caries score. A standard computer programme was used for statistical analysis (Statview; Abacus Concepts, Berkeley, CA 94704, USA).

Results

Table 1 shows the distribution of *S. mutans* and *S. sobrinus* in different age groups. In all subjects, the prevalence was 72.8% and 61.1%, respectively; 19 (24.7%) were positive for *S. mutans* alone, 10 (13.0%) were positive for *S. sobrinus* alone, 37 (48.1%) were positive for both *S. mutans* and *S. sobrinus*, and 11 (14.3%) were negative for both *S. mutans* and *S. sobrinus*.

The caries prevalence in children with only *S. mutans*, or *S. mutans* and *S. sobrinus*, as related to the age of detection, is shown in Table 2. Overall, the ft and dmft scores of children positive for both *S. mutans* and *S. sobrinus* were significantly higher than those positive for *S. mutans* alone ($p < 0.05$, $p < 0.01$, respectively). In the 3-year-old age group, the dmft score of children positive for both *S. mutans* and *S. sobrinus* was significantly higher than those positive for *S. mutans* alone ($p < 0.05$). PCR analysis with 16S rDNA primers confirmed the presence of bacteria in all plaque samples (data not shown).

Table 1. Distribution of mutans streptococci in different age groups

Organisms present		Number (%) of subjects aged			
S. mutans	*S. sobrinus*	3 years	4 years	5 years	Total
+	−	7 (31.8)	5 (20.8)	7 (22.6)	19 (24.7)
+	+	11 (50.0)	10 (41.7)	16 (51.6)	37 (48.1)
−	+	2 (9.1)	5 (20.8)	3 (9.7)	10 (13.0)
−	−	2 (9.1)	4 (16.7)	5 (16.1)	11 (14.3)
Total		22	24	31	77

Table 2. Caries prevalence in children with *S. mutans* alone or both *S. mutans* and *S. sorbinus*, related to the age at detection

		Mean (SD) dt		Mean (SD) ft		Mean (SD) dmft	
Age (years)	n	*S. mutans*	*S. mutans* + *S. sobrinus*	*S. mutans*	*S. mutans* + *S. sobrinus*	*S. mutans*	*S. mutans* + *S. sobrinus*
3	22	0.86 (1.22)	1.18 (1.17)	3.14 (3.24)	7.73 (4.82)*	4.00 (3.11)	8.91 (4.81)*
4	24	0.40 (0.89)	2.60 (3.27)	6.80 (5.89)	6.50 (4.91)	7.40 (5.73)	9.20 (4.34)
5	31	0.29 (0.76)	0.75 (1.01)	6.14 (3.53)	8.81 (4.64)	6.43 (3.60)	9.88 (4.53)
Total	77	0.53 (0.96)	1.38 (2.03)	5.21 (4.25)	7.87 (4.73)	5.79 (4.12)	9.41 (4.46)†

Statistical significance; *$p < 0.05$, †$p < 0.01$.

Table 3 shows PCR results obtained from the 13 (16.9%) children who were free from caries. Of these, two had only *S. mutans*, four had only *S. sobrinus*, one had both, and six had neither.

Discussion

Transmission of and colonisation by mutans streptococci in the oral cavity are important factors for prevention of dental caries. Most children receive the organisms from their mothers [23–26]. Furthermore, Straetemans *et al*. [27] have suggested that delayed acquisition of mutans streptococci may reduce the incidence of caries in both primary and permanent dentition at later ages. Moreover, acquisition of mutans streptococci has been found to be most common during a discrete window period that occurs at around the age of 26 months [28]. Therefore, it is of great importance to detect the presence of mutans streptococci in early years for dental caries prediction and subsequent treatment. The PCR method used to detect *S. mutans* and *S. sobrinus* with 16S rDNA primers confirmed the presence of bacteria in all positive plaque samples in the present study (data not shown). This tool provides a more sensitive means of detection of cariogenic bacterial species, as compared with conventional culture techniques [12, 15, 29].

The results of the present study indicated that the prevalence of mutans streptococci in 3-, 4- and 5-year-olds was 85.7%, 72.8% and 61.1%, respectively, which is in agreement with other surveys of pre-school children [30–32]. The percentage of children positive for only *S. mutans*, or both *S. mutans* and *S. sobrinus*, was similar in each age group. It has been suggested that these bacteria are generally established in the oral cavity of children before 3 years of age; however, the present study found that 13.0% of all the children had *S. sobrinus* only, while 48.1% had both *S. mutans* and *S. sobrinus*. Köhler *et al*. [30] reported that *S. sobrinus* was usually found in combination with *S. mutans* in 4-year-old pre-school children, except for two subjects in whom *S. sobrinus* was the only species detected. Dissimilar observations have been made in other populations [33, 34]. Also, it has been reported that mitis-salivarius bacitracin inhibits the growth of *S. sobrinus* more than that of *S. mutans* [10, 11]. The inconsistencies among these studies and the present study might be due in part to the detection methods employed or the ethnic backgrounds of the study subjects. Finally, the results of the present study suggest that the PCR method is suitable for investigation of the intra-oral distribution of *S. sobrinus* as well as *S. mutans*.

Table 3. PCR results from caries-free children

Subject no.	Sex	Age (years, months)	*S. mutans*	*S. sobrinus*	rDNA
1	Male	3,0	+	–	+
2	Male	3,1	+	+	+
3	Female	3,3	–	–	+
4	Male	3,7	–	–	+
5	Male	4,0	–	–	+
6	Male	4,1	–	+	+
7	Male	4,1	–	–	+
8	Male	4,1	–	+	+
9	Male	4,11	–	+	+
10	Male	4,11	–	+	+
11	Male	4,9	–	–	+
12	Male	5,1	–	–	+
13	Male	5,8	+	–	+

PCR results of the present study showed that children with both *S. mutans* and *S. sobrinus* had a significantly higher prevalence of caries than those with only *S. mutans*, which agrees with the results of a previous study by Köhler *et al*. [30]. They suggested that the presence of both *S. mutans* and *S. sobrinus* is associated with a significantly higher caries prevalence than *S. mutans* alone in pre-school children, although the subject population in their test was limited. Furthermore, in other reports, children with multiple species had high numbers of total mutans streptococci [30, 31, 33, 35]; however, Thomson *et al*. [36] suggested that the establishment of *S. sobrinus* in the presence of *S. mutans* occurred only in association with a high sucrose-containing diet.

In the present study there was no significant difference in the number of untreated decayed teeth between children positive for *S. mutans* alone and those with both *S. mutans* and *S. sobrinus*. Cross-sectional studies such as this one may not reveal considerable distinctions, whereas longitudinal ones may be expected to

show significant differences between dental caries levels and a combination of mutans streptococci. Furthermore, in the present findings the d (decayed) component was low, as all decayed teeth were immediately filled and therefore included in the f (filled) component during dental check-ups that occurred at intervals of every few months to 6 months. Indeed, the score of decayed teeth tended to be lower according to increasing age. Longitudinal studies are required to evaluate the acquisition and colonising combination of mutans streptococci in the oral cavity of children, and to compare the results to the incidence of dental caries.

In the present study, 13 (16.9%) of 77 children were caries-free; however, 7 (53.8%) of those had either *S. mutans* or *S. sobrinus* alone, and one had both bacterial species. Matee *et al*. [37] reported high levels of mutans streptococci in some caries-free children. They also suggested that *S. sobrinus* did not seem to play a major role in the children aged 1–2.5 years with rampant caries. Mattos-Graner *et al*. [38] suggested that the ability to synthesise water-insoluble glucan is an important virulence factor in initial caries development, in that it increases mutans streptococcal adherence and accumulation in the plaque of young children. Grönroos *et al*. [39] found that the mutacin activity of clinical isolates is reasonably stable, and this virulence factor seems to be of clinical importance in early colonisation by *S. mutans*. An understanding of the route of transmission of mutans streptococci may help clinicians to develop measures to prevent, delay, or reduce colonisation, thereby decreasing caries incidence [27, 40, 41].

In conclusion, the results of the present study indicate that children harbouring both *S. mutans* and *S. sobrinus* had a significantly higher incidence of dental caries than those positive for *S. mutans* alone.

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