Longitudinal study of dental caries incidence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in pre-school children

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Streptococcus mutans and *Streptococcus sobrinus* are known to be associated with the development of dental caries. In this study these bacteria were detected in pre-school children (each with primary dentition, age range 3–5 years, $n = 60$) using a PCR method, and then their presence was compared with the incidence of dental caries over a 1-year period. Plaque samples were collected from all erupted tooth sites using a sterile toothbrush. Dental examinations at the beginning of the study (baseline) and after 1 year were also performed to determine decayed, missing, filled teeth (dmft) scores using WHO caries diagnostic criteria. The prevalences of *S. mutans* and *S. sobrinus* across all the subjects were 61.7 % and 56.6 %, respectively; 13 subjects (21.7 %) were positive for *S. mutans* alone, 10 (16.6 %) were positive for *S. sobrinus* alone and 24 (40.0 %) were positive for both *S. mutans* and *S. sobrinus*, whereas 13 (21.7 %) were negative for both *S. mutans* and *S. sobrinus*. dmft scores of subjects positive for both *S. mutans* and *S. sobrinus* at baseline and after 1 year were significantly higher than of those positive for *S. mutans* alone at the same stages ($P < 0.01$ and $P < 0.001$, respectively). The caries incremental increase was also significantly greater in those with both bacteria detected ($P < 0.05$). Our results indicate that pre-school children harbouring both *S. mutans* and *S. sobrinus* have a significantly higher incidence of dental caries than those with *S. mutans* alone.

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INTRODUCTION

Mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) are considered to be major dental caries aetiological agents. They are the most common pathogens isolated from human dental plaque and their prevalence has been reported in epidemiological studies (Hamada & Slade, 1980; Whaley & Beighton, 1998). *S. mutans* has been shown to be more prevalent than *S. sobrinus* in dental plaque samples (Loesche, 1986; Carlsson *et al.*, 1987), while several epidemiological studies have shown that the prevalence of *S. sobrinus* is more closely associated with high caries activity (Fujiwara *et al.*, 1991; Hirose *et al.*, 1993).

In several epidemiological studies, identification of *S. mutans* and *S. sobrinus* on selective media such as mitis-salivarius (MS) or MS-bacitracin (MSB) agar has been performed using colonial morphology (Wade *et al.*, 1986; Svanberg & Krasse, 1990). However, accurate differentiation between *S. mutans* and *S. sobrinus* is not easy, and is also time-consuming and laborious (de Soet *et al.*, 1990). Further, it has been reported that *S. sobrinus* from dental plaque samples is especially difficult to culture directly on MSB selective

medium (Jordan 1986; de Soet *et al.*, 1990). 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.

Thus far, several methods used for detecting and identifying mutans streptococci have been reported, including direct microscopy, cultivation, enzyme tests, mAbs, ELISAs and species-specific DNA probes. Several investigators have also developed PCR methods and reported them to be more sensitive for detection than conventional culture techniques (Ono *et al.*, 1994; Igarashi *et al.*, 2000), as they have been shown to be able to detect low numbers of bacterial species with a detection limit of as few as 25–100 cells (Igarashi *et al.*, 1996, 2000), while being quick and relatively simple to perform. Further, PCR assays were found to be suitable for the specific detection and identification of human cariogenic bacteria, such as *S. mutans* and *S. sobrinus* (Igarashi *et al.*, 1996, 2000; Shiroza *et al.*, 1998).

In a previous cross-sectional study, we reported that children harbouring both *S. mutans* and *S. sobrinus* had a significantly higher incidence of dental caries than those with *S. mutans*

Abbreviation: dmft, Decayed, missing, filled teeth.

alone (Okada *et al.*, 2002). However, there are few longitudinal studies of the relationship between these two species and caries activities in children (Hirose *et al.*, 1993).

In the present study, we detected *S. mutans* and *S. sobrinus* using a PCR method in pre-school children, and then compared their presence with the incidence of dental caries over a 1-year period.

METHODS

Sixty Japanese pre-school children, all aged 3–5 years and with primary dentition, who were visitors to the Hiroshima University Dental Hospital were enrolled in this study. Consent for participation was obtained from at least one of their parents prior to 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 (Y. S. and T. D.) while seated in a dental chair, using the WHO caries diagnostic criteria to determine the decayed, missing, filled teeth (dmft) index (WHO, 1987). Those who had received antibiotics within the previous 3 months or with systemic diseases were excluded. In a sample group of 20 subjects, the percentage agreement between the examining dentists was over 90 % for inter-examiner reproducibility for dmft criteria.

Plaque sampling. Dental plaque was collected from all erupted teeth by brushing with a sterile toothbrush for 1 min, using a previously described method (Okada *et al.*, 2000). 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 our research laboratory and stored at –20 °C, prior to extraction of genomic DNA.

Genomic DNA preparation. *S. mutans* JCM5175^T and *S. sobrinus* ATCC27607^T were used as controls. PCR detection of the tested species was performed using primers described by Igarashi *et al.* (1996, 2000) while that of 16S rDNA was done by the method of Goncharoff *et al.* (1993).

Plaque samples were first harvested by centrifugation at 1600 g for 20 min. The supernatants were then discarded, and individual cell pellets were stored at –20 °C until DNA isolation. A genomic DNA preparation from each plaque sample was obtained using a standard miniprep procedure (Wilson, 1990), to which we added an RNase treatment (Smith *et al.*, 1989). DNA concentrations in the dental plaque samples were calculated by measuring A₂₆₀ and the quality was estimated by the A₂₆₀/A₂₈₀ ratio (Sambrook *et al.*, 1989).

PCR amplification. PCR amplification was performed in a reaction mixture (25 µl) consisting of PCR beads (Amersham Pharmacia Biotech) that contained an enzyme, along with the required reagents,

25 pmol of each primer and 20–50 ng of template DNA solution in a thermal cycler (PC-700 program temperature control system; ASTEC). Each set of PCR analyses included a negative control (water blank) in addition to the positive control. The reaction mixture was denatured at 95 °C for 3 min, followed by 26 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, with a final cycle of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 5 min (Igarashi *et al.*, 2000). Following amplification, 15 µl of the PCR products was analysed by electrophoresis on a 1·2 % agarose gel. After staining with ethidium bromide, the newly synthesized DNA fragments were visualized under a 302 nm UV light. The size of the PCR products was estimated from the electrophoretic migration of products relative to a 100 bp ladder marker (Amersham Pharmacia Biotech).

Statistical analysis. A Mann-Whitney U-test was employed to compare caries scores between combinations of the bacteria, and a Wilcoxon rank test was used to compare caries scores between the baseline and after 1 year. A standard computer program was used for statistical analysis (Statview; Abacus Concepts).

RESULTS

At baseline the prevalences of *S. mutans* and *S. sobrinus* were 61·7 % and 56·6 %, respectively. Thirteen (21·7 %) subjects were positive for *S. mutans* alone, 10 (16·6 %) for *S. sobrinus* alone and 24 (40·0 %) positive for both *S. mutans* and *S. sobrinus*, while 13 (21·7 %) were negative for both bacteria.

Table 1 shows the prevalence of previous caries experience and caries incremental increases in the children, and the combinations of the two bacteria detected in them. Twelve (92·3 %) of the children with *S. mutans* alone and 23 (95·6 %) with both *S. mutans* and *S. sobrinus* had past caries experiences. Further, five (38·5 %) of the children with *S. mutans* alone, four (40·0 %) of those with *S. sobrinus* alone and 16 (66·7 %) of those with both *S. mutans* and *S. sobrinus* had caries incremental increases, while two (15·4 %) with neither *S. mutans* nor *S. sobrinus* also had an incremental increase.

Caries prevalences in children with *S. mutans* alone or in combination with *S. sobrinus* at baseline and after 1 year are shown in Table 2. The dmft scores of those positive for both organisms at baseline and after 1 year were significantly higher than of those positive for *S. mutans* alone at the same stages (*P* < 0·01 and *P* < 0·001, respectively). Further, dmft scores of subjects positive for both after 1 year were significantly higher than at baseline (*P* < 0·001), and the

Table 1. Children with previous caries experience at baseline and caries incremental increases after 1 year, and the combination of mutans streptococci detected

Micro-organisms present		Previous caries experience		Caries incremental increase	
<i>S. mutans</i>	<i>S. sobrinus</i>	No.	%	No.	%
+	–	12	92·3	5	38·5
+	+	23	95·6	16	66·7
–	+	5	50·0	4	40·0
–	–	6	46·2	2	15·4

Table 2. Caries prevalence in children with *S. mutans* alone or in combination with *S. sobrinus* at baseline and after 1 year

Statistical significance between groups: * $P < 0.01$, ** $P < 0.001$, *** $P < 0.05$, Mann-Whitney U test; † $P < 0.001$, Wilcoxon rank test. Data for dmft scores are presented as mean (SD).

Micro-organisms present	No. at baseline	dmft		Increase in dmft
		Baseline	After 1 year	
<i>S. mutans</i>	13	4.31 (3.59)*	4.85 (3.83) **	0.54 (0.78)***
<i>S. mutans</i> + <i>S. sobrinus</i>	24	9.58 (5.23)*†	11.67 (4.82) **†	2.08 (2.03)***

increase in dmft scores of subjects positive for both *S. mutans* and *S. sobrinus* was significantly greater than in those positive for *S. mutans* alone ($P < 0.05$).

Table 3 shows PCR results obtained from the 11 (18.3%) children who were free from caries. Of these, two had only *S. mutans* and three had only *S. sobrinus*, while no children had both and six had neither detected.

DISCUSSION

Mutans streptococci transmission and colonization in the oral cavity are important factors for the prevention of dental caries. Usually the mother is regarded as the main source of the organisms found in children, based on chromosomal DNA-fingerprinting techniques used for the study of intra-family clonal distribution (Alaluusua *et al.*, 1994; Li & Caufield, 1995; Saarela *et al.*, 1996). Straetmans *et al.* (1998) suggested that a delayed acquisition of mutans streptococci may reduce the incidence of caries in both primary and permanent dentition later on. Further, acquisition of the organisms has been suggested to occur during a discrete age interval, termed the window of infectivity, between 19 and 31 months of age, during which the proportion of children with mutans streptococci was found

to increase from 25% to 75% (Caufield *et al.*, 1993; Köhler & Andréen, 1994). Thus, it is of great importance to detect the presence of these organisms in early childhood for dental caries prediction and subsequent treatment, as *S. mutans* and *S. sobrinus* are regarded as the main initiator micro-organisms of the disease, with lactobacilli and others participating in its progression. Further, the level of mutans streptococci in the oral cavity has been shown to have a correlation with both past caries experience and future caries activity (Köhler *et al.*, 1981; Newbrun *et al.*, 1984). The present results confirm that children with both *S. mutans* and *S. sobrinus* have a significantly higher caries incidence and incremental increase than those with *S. mutans* alone.

Our results suggested that the PCR method employed is suitable for investigating the intra-oral distribution of *S. sobrinus* as well as *S. mutans*, as the 16S rDNA primers used confirmed the presence of bacteria in all plaque samples (data not shown). This tool provides a more sensitive means of detection of cariogenic bacterial species, as compared to conventional culture techniques (Ono *et al.*, 1994; Igarashi *et al.*, 1996, 2000).

The present findings showed that the prevalence of mutans streptococci in subjects 3–5 years old was 78.3%, which is in

Table 3. PCR results from children who were caries-free after 1 year

Subject no.	Sex	Age at 1-year stage (years, months)	PCR result		
			<i>S. mutans</i>	<i>S. sobrinus</i>	rDNA*
1	Male	4, 11	—	—	+
2	Male	5, 0	—	+	+
3	Female	4, 8	+	—	+
4	Female	5, 2	—	—	+
5	Female	4, 2	—	—	+
6	Male	4, 4	+	—	+
7	Male	5, 1	—	—	+
8	Female	4, 1	—	+	+
9	Male	4, 9	—	+	+
10	Male	4, 3	—	—	+
11	Female	4, 1	—	—	+

*16S ribosomal DNA.

agreement with other surveys of pre-school children (Köhler *et al.*, 1988, 1995a; Li *et al.*, 1994). The percentages of 3–5-year-old children positive for *S. mutans* and *S. sobrinus* were 61·7 % and 56·6 %, respectively, while 16·6 % of all subjects had *S. sobrinus* only and 40·0 % had both *S. mutans* and *S. sobrinus*. Köhler *et al.* (1988) reported that *S. sobrinus* was found in combination with *S. mutans* in nearly all 4-year-old pre-school children tested, except for two in whom *S. sobrinus* was the only species detected, although contradicting results have been found with other populations (Masuda *et al.*, 1979; Köhler *et al.*, 1995b). In addition, it has been reported that MSB inhibits the growth of *S. sobrinus* to a greater degree than that of *S. mutans* (Jordan, 1986; de Soet *et al.*, 1990). The inconsistencies among those studies and ours could be due, in part, to the detection methods employed or ethnic backgrounds of the study subjects.

The present PCR findings confirmed that children with both *S. mutans* and *S. sobrinus* had a significant caries prevalence as compared to those with only *S. mutans* both at baseline and after 1 year, which agrees with the results of previous studies (Köhler *et al.*, 1988; Hirose *et al.*, 1993; Nie *et al.*, 2002). In addition, approximately 70 % of the children with both bacteria had incremental caries increases over the 1-year study period, whereas less than 40 % of those with only one of the organisms demonstrated such incremental increases. Similar results were reported in the longitudinal studies by Hirose *et al.* (1993) and Straetmans *et al.* (1998). Hirose *et al.* (1993) suggested that the prevalence of *S. sobrinus* in saliva was more closely associated with future caries activity, especially with smooth-surface caries increment, than the prevalence of *S. mutans*. In addition, a caries incremental increase was seen in a maximum of 79 % of the children in that study and the dmft index increase was approximately 2. In our study, the caries incremental increase in children with both *S. mutans* and *S. sobrinus* was four times higher than in those with *S. mutans* alone, indicating that children with both bacteria have higher caries activities than those with only *S. mutans*.

In the present study, 11 (11·8 %) of the 60 children were caries free for 1 year, of whom five (45·5 %) had either *S. mutans* or *S. sobrinus*, while none had both bacterial species. As a result, it is strongly suggested that the presence of both *S. mutans* and *S. sobrinus* in pre-school children is associated with significantly higher caries incidence than *S. mutans* alone. However, since the subject population in the present study was limited, further studies are required.

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

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