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# Quantitative real-time polymerase chain reaction for *Streptococcus mutans* and *Streptococcus sobrinus* in dental plaque samples and its association with early childhood caries

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**Background.** *Streptococcus mutans* and *Streptococcus sobrinus* are closely associated with the development of early childhood caries (ECC). Recently, quantitative real-time polymerase chain reaction (qRT-PCR) has been used for rapid and accurate quantification of these bacterial species.

**Aim.** This study aims to detect quantitatively the levels of *S. mutans* and *S. sobrinus* in plaque samples by qRT-PCR, and to assess their association with the prevalence of ECC in Korean preschool children.

**Design.** One hundred and five children (71 months old or younger) were examined and classified into three groups (caries-free, ECC, severe ECC). Dental plaque samples were collected and qRT-PCR was conducted using oligonucleotide primers specific for glucosyltransferase gene (*S. mutans-gtfB*, *S. sobrinus-gtfU*) and universal primer. Pearson's correlation test was conducted to evaluate the relationship between the dmfs (decayed, missing, or filled surfaces primary teeth) scores and the microbiological findings.

**Results.** There was a significant difference between the levels of *S. mutans* and *S. sobrinus* in the plaque samples of the three groups ($P < 0.05$). The proportion of *S. sobrinus* to *S. mutans* showed strong correlation to the dmfs scores ($r = 0.748$, $P < 0.05$).

**Conclusions.** The qRT-PCR results of this study showed that children with ECC had higher level of *S. mutans* and *S. sobrinus* in their dental plaque samples. The children with higher ratio of *S. sobrinus* to *S. mutans* in their dental plaque showed higher incidence of ECC.

## Introduction

*Streptococcus mutans* and *Streptococcus sobrinus* are mutans streptococci mainly found in human dental plaque, and are strongly associated with the development of dental caries. The levels of *S. mutans* in the oral cavity has been shown to have a correlation with both past caries experience and future caries activity[1,2]. Although *S. sobrinus* is less frequently detected than *S. mutans*, several studies have reported that the prevalence of *S. sobrinus* was more closely associated than *S. mutans* with caries activity, especially with smooth-surface caries increment[3,4]. Caries increment in children with both *S. mutans* and *S. sobrinus* was four times higher than in those with *S. mutans* alone, and *S. sobrinus* had higher acidogenic capacity compared to *S. mutans*[5–7]. However, the significance of the relative numbers of these bacteria has been unclear, due to the lack of methods to accurately differentiate and quantify them. Various methods have been used for detection of mutans streptococci, including culture, direct enzyme tests, and enzyme-linked immunosorbent assay. Most of these methods are time-consuming, laborious, and relatively unspecific. End-point polymerase chain reaction (PCR) has also been used for the detection of mutans streptococci in previous studies[8,9]. Although conventional end-point PCR is highly specific and sensitive compared to other methods, it lacks the ability for precise quantification.

Quantitative real-time PCR (qRT-PCR) with species-specific primers can provide an accurate and sensitive method for detection and quantification of individual species and bacterial populations as well as total bacteria[10,11]. The ability to quantify the bacteria in a sample has advantages to previous approaches in that not only presence or absence but rather the

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amount of bacteria can be related to clinical conditions. In qRT-PCR, amplification, measurement, and quantification of PCR product occur simultaneously in the same closed reaction tube. Thus, the need for post-PCR manipulation is obviated and the risk of PCR product carryover contamination is minimized, which is a big advantage compared with end-point PCR[12]. In qRT-PCR, the bacteria does not have to be alive, and samples remain stable over long periods of freezing. Easy sample handling can be a benefit for large-scale screening[13]. In addition, qRT-PCR assay has a very large dynamic range of target molecule determination because the real-time measurement of the PCR product allows to quantify the amplified products in the log phase of the reaction.

The purpose of this study was to use qRT-PCR for quantification of *S. mutans* and *S. sobrinus* in dental plaque samples of Korean preschool children, and compare the microbiological data with individual's dmfs score to find an association with early childhood caries.

## Materials and methods

### *Subjects and plaque sampling*

Prior to the initiation of the study, the single dental examiner was trained to ensure consistency in examination and caries diagnosis. The kappa values for intra-examiner agreement for caries was 0.95. The presence of decayed, missing, or filled surfaces was scored according to World Health Organization[14]. Teeth were considered decayed if there was an unmistakable cavity, undermined enamel, or a detectably softened floor or wall. White spots were coded as sound. Radiographs were exposed only when there was any doubt if a child had proximal caries or not.

One hundred and five healthy children (71 months old or younger) who visited Seoul National University Dental Hospital were examined and classified into three groups (CF, caries-free; ECC, early childhood caries; SECC, severe early childhood caries) according to American Academy of Pediatric Dentistry criteria[15]. Children were coded as CF when no caries were present and ECC when there were more than 1 dmfs in any primary tooth. SECC was coded when a child younger than 3 years had any sign of smooth surface caries, when 3- to 5-year-old child had more than 1 dmfs in primary maxillary anterior teeth, or when there were more than 4, 5, and 6 dmfs in a 3-, 4-, and 5-year-old child, respectively.

All the children were in good general health. Those who had received any antibiotic therapy during the last 2 weeks or fluoride topical application during the last 48 h were excluded from the study. The investigation received approval from the Institutional Review Board of Seoul National University Dental Hospital (Investigation No. CRI06009), and informed consent was obtained from the parents. Supragingival plaque samples were collected from sound buccal surfaces of maxillary primary molars with sterile dental explorers. If all the sampling sites were decayed, the individual was excluded from the study. A small visible amount of plaque was transferred to a sterile 1.7 mL tube, and stored at –20 °C until further analysis. Each sample was weighed individually with a micro balance (Sartorius, Goettingen, Germany).

### *Real-time PCR*

The bacterial strains *S. mutans* Ingbritt and *S. sobrinus* 6715-7 used in this study were obtained from Seoul National University Dental Research Center Culture Collection (Seoul, South Korea). Bacterial DNAs were extracted from bacterial pellet obtained by centrifugation of 10 mL of overnight culture and clinical plaque samples by using G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology Inc., Sungnam, South Korea) according to the manufacturer's instructions. Tenfold serial dilutions of the bacterial suspension were made, and the resulting dilutions were independently enumerated with a Petroff-Hausser counter chamber several times independently by two persons. To generate a standard curve for real-time PCR, the bacterial DNA was extracted from the different dilutions and the concentration was adjusted; 1 μL of DNA thus corresponded to a defined number of CFU. The threshold cycle ($C_T$) values at the different dilution points were averaged. The total number of cells was interpolated from the averaged standard curve. For quantification

**Table 1. Oligonucleotide primers used in this study.**

| | Primer | Sequence | Position | Amplicon size | Reference |
|---|---|---|---|---|---|
| *gtfB* | F | 5′-CTACACTTTCGGGTGGCTTG-3′ | 794-813 | 261 bp | Fujiwara *et al.*[16] |
| | R | 5′-GAAGCTTTTCACCATTAGAAGCTG-3′ | 1054-1031 | | |
| *gtfU* | F | 5′-AAAACATTGGGTTACGATTGCG-3′ | 39-60 | 156 bp | This study |
| | R | 5-CGTCATTGGTAGTAGCCTGA-3′ | 193-174 | | |
| Universal | F | 5′-TGGAGCATGTGGTTTAATTCGA-3′ | 930-951 | 160 bp | Sinsimer *et al.*[17] |
| | R | 5′-TGCGGGACTTAACCCAACA-3′ | 1089-1071 | | |

of total bacteria, standard curves from 10 different bacterial strains were generated with real-time PCR, and linearity with the universal primer was found.

Species-specific primers for *S. mutans* and *S. sobrinus* were designed (Bioneer, Daejun, South Korea) from the *gtfB* and *gtfU* genes, respectively (Table 1)[16]. In order to quantify total bacteria, conserved region was selected in the 16 s rRNA[17]. To test the specificity of the primers, 10 bacterial species found most frequently in the oral cavity (*Streptococcus gordonii* $DL^{-1}$, *Streptococcus mutans* Ingbritt, *Streptococcus rattus* BHT, *Streptococcus sanguinis* 804, *Streptococcus sobrinus* 6715-7, *Actinobacillus actinomycetemcomitans* ATCC 33384, *Fusobacterium nucleatum* ATCC 10953, *Prevotella intermedia* ATCC 49046, *Porphyromonas gingivalis* ATCC 33272, and *Escherichia coli* DH5α) were used as templates for conventional PCR with *gtfB*, *gtfU*, and universal primers. The PCR products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide dye, and detected by LAS-1000 (Fujifilm, Tokyo, Japan).

Real-time PCR was performed with GeneAmp 7500 Sequence detection system (PE Applied Biosystems, Foster City, CA, USA). two μe of template DNA and 0.4 μe of primers were mixed with 0.4 μe of ROX dye and 10 μe of SYBR® *Premix Ex Taq*™ (Takara, Shiga, Japan). Distilled water was added to make the final volume of 20 μe. Amplification was carried out as follows: initial denaturation for 4 min at 94 °C, 40 cycles of denaturation for 20 s at 94 °C, primer annealing for 20 s at 55.4 °C, 57.5 °C, 56.7 °C for *gtfB*, *gtfU*, universal primers, respectively, and extension for 33 s at 72 °C. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical caps (PE Applied Biosystems), and were performed three times repeatedly. Bacterial amount in 1 mg of plaque sample was obtained by dividing the bacterial load by plaque weight (mg).

### *Statistical analysis*

Statistical evaluation was performed by one-way analysis of variance with SPSS version 12.0 (SPSS, Chicago, IL, USA). Statistical significance was defined as $P \leq 0.05$. Pearson's correlation test was used to evaluate the relationship between the clinical data and the microbiological findings.

## Results

The specificity of the real-time PCR assay was investigated with conventional PCR (Fig. 1).

A standard curve for bacterial quantification was fabricated using 10-fold serial dilutions of *S. mutans* and *S. sobrinus* (Fig. 2a,b). Evaluation of the primer pair to enumerate total bacteria was performed with serial dilutions using 10 different bacterial species described above. PCR quantification of total bacteria was not restricted to one species and universal primer detected all tested species (data not shown). Dissociation curves showed a sharp peak at the expected $T_m$ of the products, indicating that each real-time PCR specifically amplified the target DNA (data not shown).

*Streptococcus mutans* was detected in 80% of the CF group ($n = 28$) and in 100% of the ECC and SECC groups. *S. sobrinus* was detected less frequently than *S. mutans*. *S. sobrinus* was detected in 8.6% ($n = 3$), 42.9% ($n = 15$), and 60.0% ($n = 21$) in the CF, ECC, and SECC groups, respectively. The mean dmfs scores were 0, 3, and 10.6 in the CF, ECC, and SECC groups, respectively.

The levels of *S. mutans*, *S. sobrinus*, and total bacteria in plaque samples of 105 children

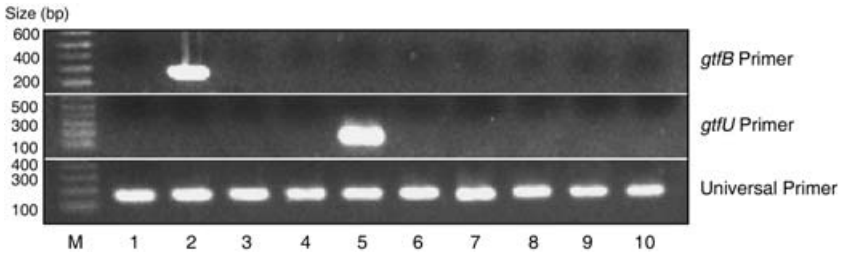


**Fig. 1.** Evaluation of the specificity of the *gtfB*, *gtfU*, and universal primers by conventional polymerase chain reaction amplification. All bacterial genomic DNA showed amplification with universal primer. *gtfB* primer pair showed amplification only when *Streptococcus mutans* DNA was used as template, and *gtfU* primer pair showed amplification with *Streptococcus sobrinus* DNA only (M, size marker; 1, *Streptococcus gordonii*; 2, *S. mutans*; 3, *Streptococcus rattus*; 4, *Streptococcus sanguinis*; 5, *S. sobrinus*; 6, *Actinobacillus actinomycetemcomitans*; 7, *Fusobacterium nucleatum*; 8, *Prevotella intermedia*; 9, *Porphyromonas gingivalis*; and 10, *Escherichia coli*).

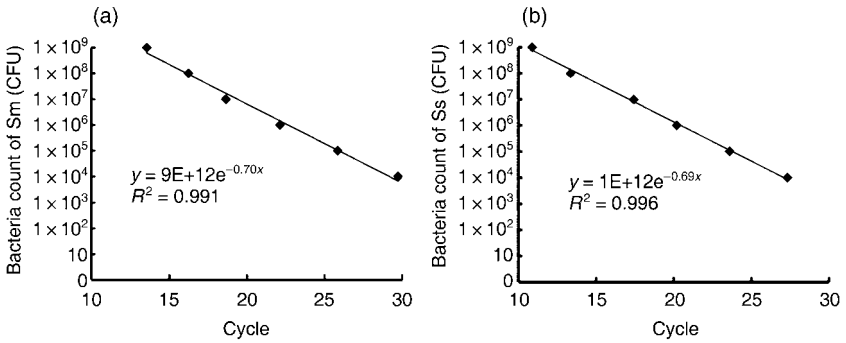


**Fig. 2.** Standard curves of the real-time polymerase chain reaction assays for bacterial quantification, constructed using the serial dilutions of pure bacterial culture. The horizontal axis of panels indicate the threshold cycles (cycle number when the fluorescence reached threshold line). The vertical axis indicates cell number of *Streptococcus mutans* and *Streptococcus sobrinus*. (a) Standard curve of *S. mutans*; (b) standard curve of *S. sobrinus*.

were determined by conversion of the $C_T$. The ratio of *S. mutans* : Total bacteria, *S. sobrinus* : Total bacteria, and *S. sobrinus* : *S. mutans* were determined as well (Table 2).

PCR results from dental plaque samples were correlated to dmfs index, using Pearson's correlation test. The detected *S. sobrinus* in 1 mg of dental plaque was significantly correlated with the dmfs scores, and the ratio of *S.mutans* : Total bacteria and *S. sobrinus* : Total bacteria also showed moderate correlation with the dmfs scores. A significant high correlation was found between the ratio of *S. sobrinus* : *S. mutans* and the dmfs scores (Table 2).

## Discussion

The results of this study showed that children with ECC had a significantly higher level of mutans streptococci in their dental plaque

**Table 2. Counts and ratio of bacterial cells in plaque samples, and Pearson's correlation between dmfs and microbiological findings.**

| | | Total | Sm* | Ss* | Sm/Total* | Ss/Total* | Ss/Sm* |
|---|---|---|---|---|---|---|---|
| CF | | $6.0 \times 10^7 \pm 8.7 \times 10^6$ | $1.8 \times 10^4 \pm 4.4 \times 10^5$ | $3 \pm 1.17$ | 0.04% | 0.00% | 0.00% |
| ECC | | $7.2 \times 10^7 \pm 8.7 \times 10^6$ | $2.9 \times 10^5 \pm 2.1 \times 10^5$ | $9.9 \times 10^3 \pm 2.7 \times 10^3$ | 0.9% | 0.03% | 0.04% |
| SECC | | $5.4 \times 10^7 \pm 6.6 \times 10^6$ | $1.9 \times 10^6 \pm 1.6 \times 10^5$ | $5.1 \times 10^5 \pm 8.6 \times 10^5$ | 8% | 1.60% | 0.26% |
| dmfs | *r* | –0.044 | 0.211* | 0.437** | 0.301* | 0.344** | 0.748* |

Bacterial counts: mean ± standard error. $r$ = Pearson's correlation coefficient ($0.1 < r < 0.3$: low correlation; $0.3 < r < 0.7$: moderate correlation; $0.7 < r < 1.0$: high correlation). *$P < 0.05$; **$P < 0.01$.

samples. Although *S. sobrinus* was detected less frequently and in lower numbers than *S. mutans*, the proportion of *S. sobrinus* to *S. mutans* showed highest correlation with children's dmfs. So far, the relationship between *S. sobrinus* level in dental plaque and caries incidence has not been explored due to difficulties in detection and quantification of *S. sobrinus*. Recently, the application of the PCR method to human dental plaque showed that the prevalence of *S. sobrinus* (83%) in oral cavities was higher than currently supposed (0–50%)[8]. Since there have been studies about the significance of *S. sobrinus* in children with ECC, the authors thought that it would be of interest to investigate whether there is an association between absolute or relative numbers of *S. sobrinus* in dental plaque and caries incidence.

In this study, we chose qRT-PCR for bacterial quantification because it is the most accurate and reliable tool to determine bacterial concentrations in clinical samples. With qRT-PCR, a broad range of bacterial cells can be measured. The specificity of detection in the PCR can be provided by specific primers amplifying species-specific target sequence. In our study, *gtfB* and *gtfU* genes were used for designing PCR primers. *gtfB* and *gtfU* genes express important virulence factors of mutans streptococci, glucosyltransferase. *gtfB* and universal primers have been designed as described in other studies, and *gtfU* primer was designed by us from GenBank sequence database[16,17].

Hata *et al*. have reported that the ratio of *S. mutans* count to total bacterial count was higher in children with dental caries[11]. Similarly, the ratio of *S. mutans* to total bacterial count was significantly higher in the ECC and SECC groups in our study. However, the results from correlation test showed that ratio of *S. sobrinus* : total bacteria or ratio of *S. sobrinus* : *S. mutans* had higher correlation with children's dmfs. This might suggest that *S. sobrinus* is more related to ECC than previously thought. Rodriguez reported that *S. sobrinus* was detected twice as much in caries active group, and dmfs index was positively correlated with *S. mutans* and *S. sobrinus*, which coincides with our results[18,19]. Ahmady *et al*. have reported that the dmft index was higher in the children detected with both *S. mutans* and *S. sobrinus* than in the children detected with *S. mutans* only[3]. In the 3- to 5-year-old group, the ratio of children detected with *S. mutans* only was 25% and children with both *S. mutans* and *S. sobrinus* was 48%, and there were 10% of children without detected *S. mutans* in their saliva[6,20]. In this study, *S. mutans* was detected in 80% of children without dental caries, and in 100% of children with dental caries, which showed higher detection rates than those of previous studies. *S. sobrinus* was also detected more frequently than in the previous studies. In caries-free group, *S. sobrinus* was almost not detectable, but it was detected in 43–60% of children with ECC. This might be signifying the higher sensitivity of real-time PCR assay. Since *S. mutans* is detected in most of the children, qualitative detection or semi-quantitative detection of *S. mutans* only might be of less clinical use.

Some investigators reported that *S. sobrinus* does not seem to have a great association with rampant caries in children between 1 and 2.5 years[21]. Becker *et al*. reported that *S. sobrinus* did not indicate a major role in ECC. *S. sobrinus* was detected in only 9 of 30 subjects with severe ECC[22]. In our study, *S. sobrinus* showed higher correlation with prevalence of ECC. This could be attributed to lack of sensitive isolation and detection methods for *S. sobrinus* in the past, and differences in the type and stage of subjects' caries lesions.

The main limitation of this study is that we collected plaque samples from healthy sites only. The bacterial composition could have been different if samples from carious sites were included. In addition, since the qRT-PCR is very sensitive, the sampling procedure could greatly affect the results. Using dmfs to represent caries prevalence might be another problem. Higher dmfs does not necessarily mean higher caries activity or risk. There were children who had high dmfs because of high filled score, or high decayed score. The former might represent high caries history, and the latter, high caries activity.

Detection and quantification of *S. mutans* and *S. sobrinus* in the oral cavity is important because these bacteria are relevant for diagnosis and treatment planning of dental caries, which is an infectious disease. Although salivary mutans

streptococci counts are inferior as caries predictor compared to past caries experience, many studies reported an improvement of caries prediction when oral MS counts were combined with past caries experience variable[23,24]. The results of this study could be used in conjunction with other caries risk factors for diagnosis and prevention of ECC. Moreover, the simple sample storage and rapid analysis could be a big advantage for mass screening, as in epidemiological studies or school prevention programs.

**What this paper adds**

- qRT-PCR using oligonucleotide primers proved to be a sensitive and rapid method for quantification of bacteria associated with ECC.
- The proportion of *S. sobrinus* to *S. mutans* in children's dental plaque showed high correlation with caries prevalence.

**Why this paper is important to paediatric dentists**

- Quantification of mutans streptococci may help clarify the potential role of these bacteria and may be useful for understanding complex aetiology of caries.
- qRT-PCR could be used for fast and sensitive detection and quantification of *S. mutans* and *S. sobrinus*.

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## References

1 Köhler B, Pettersson B, Bratthall D. *Streptococcus mutans* in plaque and saliva and the development of caries. *Scand J Dent Res* 1981; **89**: 19–25.
2 Newbrun E, Matsukubo T, Hoover C, *et al*. Comparison of two screening tests for *Streptococcus mutans* and evaluation of their suitability for mass screenings and private practice. *Community Dent Oral Epidemiol* 1984; **121**: 325–331.
3 Ahmady K, Marsh PD, Newman HN, Bulman JS. Distribution of *Streptococcus mutans* and *Streptococcus sobrinus* at sub-sites in human approximal dental plaque. *Caries Res* 1993; **27**: 135–139.
4 Hirose H, Hirose K, Isogai E, Miura H, Ueda I. Close association between *Streptococcus sobrinus* in the saliva of young children and smooth-surface caries increment. *Caries Res* 1993; **27**: 292–297.
5 Okada M, Soda Y, Hayashi F, *et al*. PCR detection of *Streptococcus mutans* and *S. sobrinus* in dental plaque samples from Japanese pre-school children. *J Med Microbiol* 2002; **51**: 443–447.
6 Okada M, Soda Y, Hayashi F, *et al*. Longitudinal study of dental caries incidence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in pre-school children. *J Med Microbiol* 2005; **54**: 661–665.
7 de Soet JJ, Toors FA, de Graaff J. Acidogenesis by oral streptococci at different pH values. *Caries Res* 1998; **23**: 14–17.
8 Igarashi T, Yamamoto A, Goto N. PCR for detection and identification of *Streptococcus sobrinus*. *J Med Microbiol* 2000; **49**: 1069–1074.
9 Igarashi T, Ichikawa K, Yamamoto A, Goto N. Identification of mutans streptococcal species by the PCR products of the *dex* genes. *J Microbiol Methods* 2001; **46**: 99–105.
10 Corless CE, Guiver M, Borrow R, Edwards-Jones V, Kaczmarski EB, Fox AJ. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. *J Clin Microbiol* 2000; **38**: 1747–1752.
11 Hata S, Hata H, Miyasawa-Hori H, Kudo A, Mayanagi H. Quantitative detection of *Streptococcus mutans* in the dental plaque of Japanese preschool children by real-time PCR. *Lett Appl Microbiol* 2006; **42**: 127–131.
12 Nonnenmacher C, Dalpke A, Rochon J, Flores-de-Jacoby L, Mutters R, Heeg K. Real-time polymerase chain reaction for detection and quantification of bacteria in periodontal patients. *J Periodontol* 2005; **76**: 1542–1549.
13 Yano A, Noboru K, Ida H, Yamaguchi T, Hanada N. Real-time PCR for quantification of *Streptococcus mutans*. *FEMS Microbiol Lett* 2002; **217**: 23–30.
14 World Health Organization. *Oral Health Surveys: Basic Methods*, 4th edn. Geneva, Switzerland: WHO, 1997.
15 Definition of Early Childhood Caries. Council on Clinical Affairs. http://www.aapd.org/media/policies_guidelines/d_ecc. pdf.
16 Fujiwara T, Hoshino T, Ooshima T, Hamada S. Differential and quantitative analyses of mRNA expression of glucosyltransferases from *Streptococcus mutans* MT8148. *J Dent Res* 2002; **81**: 109–113.
17 Sinsimer D, Leekha S, Park S, *et al*. Use of a multiplex molecular beacon platform for rapid detection of methicillin and vancomycin resistance in *Staphylococcus aureus*. *J Clin Microbiol* 2005; **43**: 4585–4591.
18 Rodrigues J, Martinez R, Ferreyra B, *et al*. Distribution of *Streptococcus mutans* and *Streptococcus sobrinus* in saliva of Mexican preschool caries-free and caries-active children by microbial and molecular (PCR) assays. *J Clin Pediatr Dent* 2007; **32**: 121–126.
19 van Houte J. Role of micro-organisms in caries etiology. *J Dent Res* 1994; **73**: 672–681.
20 Tanner AC, Milgrom PM, Kent R Jr. The microbiota of young children from tooth and tongue samples. *J Dent Res* 2002; **81**: 53–57.
21 Matee MI, Mikx FH, Maselle SY, Van Palenstein Helderman WH. Mutans streptococci and lactobacilli in breast-fed children with rampant caries. *Caries Res* 1992; **26**: 183–187.
22 Becker MR, Paster BJ, Leys EJ, *et al*. Molecular analysis

of bacterial species associated with childhood caries. *J Clin Microbiol* 2002; **40**: 1001–1009.

23 Klock B, Krasse B. A comparison between different methods for prediction of caries activity. *Scand J Dent Res* 1979; **87**: 129–139.

24 Alaluusua S, Kleemola E, Gronroos L, Evalahti M. Salivary caries-related tests as predictors of future caries increment in teenagers. A three-year longitudinal study. *Oral Microbiol Immunol* 1990; **5**: 77–81.