spaP gene of Streptococcus mutans in dental plaque and its relationship with Early Childhood Caries

**ABSTRACT**

Streptococcus mutans and Streptococcus sobrinus are the main pathogens associated with the development of dental caries in humans. Recently, the real-time polymerase chain reaction (qPCR-TR) has been used for fast and exact quantification of these bacteria species. This molecular biology method has made the detection of these bacteria in saliva and dental plaque possible; additionally, it aids the development of illness risk prediction. The purpose of this prospective, analytic, transversal, observational and unicenter study was to quantify the spaP gene of the Streptococcus mutans and its correlation with caries in a group of children using isolated DNA from plaque samples processed through qPCR-TR, using specific oligonucleotides for this gene detection.

**Materials and methods**

The cariogenic potential of Streptococcus mutans in the dental plaque was analysed in a group of patients aged 12 to 46 months. A descriptive statistical analysis was performed. The Spearman’s correlation coefficient was used to establish the correlation between caries (dmft) index (decayed/missing/filled primary teeth), spaP gene and age group. The Wilcoxon test was used to compare MSB cultivation technique and qPCR-TR.

**Results and conclusion**

In the molecular trials, a close association between caries prevalence in childhood and the presence and high proportion of the spaP gene of S. mutans was found. The average caries prevalence was 3.71, and it increased as age range increased. The highest caries prevalence was observed in female patients and in the oldest age range studied (40–46 months) which contrasts with the 12-18 months age that had a caries (dmft) index of zero. The amplification using as initiator the gene spaP of the nucleic acids extracted from the S. mutans resulted positive in 91.3% of the cases. Every child with caries was positive for the spaP and only 8.75% were negative, this group included children without caries. In conclusion, there was a correlation with infant caries prevalence and S. mutans.

**Keywords:** Dental plaque; Early Childhood Zaries; PCR; Streptococcus mutans.

**Introduction**

Dental caries is one of the most common infectious diseases in humans and it is primarily due to isolated microorganism groups in the dental plaque, especially Streptococcus mutans and Streptococcus sobrinus [Hamada and Slade, 1980; Loeshe, 1986], which play a major role. However, because of the multifactorial nature of caries, the presence of carbohydrates (diet), bacteria (dental plaque) and a susceptible dental surface (host) is required.

During the last years, several microbiological methods have been developed to detect streptococcus strains with cariogenic potential that include microbiological cultures, direct enzyme analysis and enzyme related immunological trials [Choi et al., 2008]. Many of these methods, however, are imprecise, time consuming and complicated, especially when anaerobic microorganisms are studied.

A recent alternative is the polymerase chain reaction technique, especially quantitative real-time procedures (qPCR-RT). This new tool provides a more sensitive and specific detection compared to conventional culture methods through the use of blank species initiators, and the possibility of bacterial DNA quantification and thus the number of bacteria [Corless et al., 2000; Hata et al., 2006].

Morawinski and Kuramitsu [1992] and Colby et al. [1995] designed DNA probes that produce hybrids of Glucosyltransferases (gtfb), and precursor to extracellular antigen A (Wap-A) genes of S. mutans. Ono et al. [1994] and Colby et al. [1995] designed PCR initiator for spaP and Wap-A genes, respectively. The S. mutans strains produce surface protein antigens I/Ii (Ag I/Ii) which might increase their ability to colonise surfaces [Lee, 1989; Gibbons, 1986].

The coding gene for Ag I/Ii (also termed spap or pac) has been cloned from two serotype c and one serotype f strains of S. mutans [Okaashi et al., 1989]. The tipification of mutations produced by S. mutans is of great importance because they represent an epidemiologic marker to establish the source of infection and transmission mechanism due to a predominant producer type of bacteriocins on and individual. Additionally, mutation production has been related with caries producing capacity [Baca et al., 1990].

The aim of this study was to quantify the spaP gene of S. mutans using the polymerase real-time chain reaction (qPCR-RT) in dental plaque samples and its relationship with Early Childhood Caries.

**Materials and methods**

**Population**

This study was performed on a group of children attending the Child Development Center Alfa Gady...
(Monterrey, Nuevo León, México). The total number of children between 12 and 46 months of age registered during the academic year 2009-2010 was of 92. Out of them, 83 complied with the inclusion criteria, but only 80 participated in the study. Patients who had received a caries preventive treatment, antibiotic treatment in the last 8 weeks before the sampling, with a syndrome or craniofacial malformations or those whose central inferior and superior incisors were absent were excluded from the study. All children were in good general health.

Parents signed the informed consent to the participation of their children in the study, which was approved by the ethics committee of the University Autónoma of Nuevo León, Monterrey Nuevo Leon, Mexico. The population was divided into two groups: patients with early childhood caries and healthy controls.

Dental plaque samples
The sampling areas were dried using cotton and, with sterile plastic sticks, a visible amount of supragingival plaque was collected from the smooth surface of the primary superior and inferior incisors. The samples were then introduced in a reduced fluid transport (RTF) with pH7 [Syved et al., 1972] and were transported in ice to the laboratory.

Caries index
Dental explorations were performed in the classrooms of the center in areas with good illumination. Before sampling, the examiner underwent calibration tests with an expert [WHO, 1997] in order to achieve agreement greater than 90%. All samples were taken by the same examiner.

DNA extraction
For the spaP of S. mutans DNA fragment amplification, the primers were selected on the base of the NGS spaP gene of S. mutans to obtain a 192 bp fragment [Ma et al., 1991]. The bacterial DNA extraction of the ATCC 33478 (GenBank: AB355620.1) strain of S. Sobrinus was performed as a negative control. The oligonucleotide primers used in this study are described in Table 1.

The nucleic acid extraction was performed using a High Pure PCR, Template Preparation Kit (Roche Applied Science, Mannheim, Germany) following the product specification with small modifications, as follows.

Before starting the extraction, the samples were defrosted by keeping them on an ice bath for 15 minutes. Once liquefied, they were homogenised and 100 µL was transferred to a new tube to which 20 µL of lysisomize was added and incubated at 37°C for 10 minutes. Then, 200 µL of Binding buffer and 60 µL of protase K were incubated for 20 min at 70°C. Finally, 200 µL of isopropanol were added and the mixture was shaken until it was completely homogenous.

The tube content was centrifuged for one minute at 10,000 rpm. Then, 500 µL of Inhibitor Removal buffer was added and centrifuged again for a minute at 10,000 rpm. The process was repeated with 500 µL of Wash buffer twice. Then, 200 µL of Elution buffer preheated to 70°C was added and the mixture was centrifuged for a minute at 12,000 rpm to obtain the DNA elution.

Finally, the purity, clarity and quantity of the elution was measured with UV spectrophotometry at 260/280 NM using a Bio-Rad spectrophotometer. Dilutions of 1:100 of the DNA with Mili-Q sterile water were made for this procedure. Once this test was performed, the samples were preserved at -20°C for future analysis.

Standard curve and DNA amplification qPCR-RT
The amplification and absolute quantification of specific S. mutans nucleic acids was performed with SYBR-Green I as detection method using the reaction mixture LightCycler 480 SYBR-Green Master (Roche).

The reaction mixture was made of the following component concentrations: 3 mM MgCl2; 10 mM Tris-HCl, pH 8; 100 mM KCI; 10 µM EDTA, 1mM DDT; 1% glycerol; 0.5% Tween®20 and 0.5% Nonidet®-P40. The reaction took place in a thermocycler Lightcycler 480 with a block for 96 wells (Roche).

The amplification programme used to generate the standard curves is described in Table 1. The absolute quantification programme to determine the bacterial load in the patient used the same conditions, with an increasing number of cycles up to 65 as the only difference. These aimed to assure that all samples were amplified even with low microorganism concentrations.

The reaction starters were designed based on the codification sequence of DNA for antigen VII, P1, spaP or PAC of the Streptococcus mutans serotype C (GenBank:17390) (Table 1).

To obtain the standard curves, the generated amplicons of a previous qPCR were broken. Spectrophotometry was used to quantify and determine the concentration in nanograms (ng) and, considering the change length and its composition, the number of copies per nanogram or volume of solution was calculated. The result was adjusted to 1 X 1011 copies/mL and then serial dilutions from 1:10 to extinction were made. The positive control used was the DNA of ATCC 700611 strain, UA 130 serotype of S. mutans and as negative control ATCC 27607 strain of S. sobrinus, and water as a blank. The S. mutans level in the dental plaque samples was determined using the calculated curve and extrapolation of the Cp. The amplification detection was made through electrophoresis in agarose gel.

Reproducibility
The PCR condition was standardised with an initial study to ensure the feasibility. In all the extractions, a positive and a negative control were used to ensure absence of contamination with other nucleic acids.

Statistical analysis
The software SPSS Inc. V. 17.0 was used to obtain the descriptive statistics of the studied variables, with special emphasis in central tendency and dispersion measures. In addition, frequency analysis was made for age range,

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<th>Initiator Sequence</th>
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<tr>
<td>spaP (F) 5'-AAC GAC CGC TCT TCA GCA GAT ACC-3' 3668-3698 192bp</td>
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<tr>
<td>spaP (R) 5'-AGA AAG AAC ATC TCT AAT TTC TTG-3' 3835-3859</td>
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Oligonucleotide sequences were calculated using gene blank number 17390 (spaP)

TABLE 1 - Oligonucleotide initiator used in this study.
The Spearman’s correlation coefficient was used to calculate the correlation between clinical and microbiological variables. The Wilcoxon test was used to compare the culture techniques MSB and qPCR-TR.

Results

The study group had 80 children, 35 were boys (43.8%) and 45 girls (56.3%). The age range was 12-46 months, the mean was 30.15 ± 8.967 months, and they were distributed in five groups according to age (table 3). Caries prevalence of the study group was 38.75%, where 10 were boys and 2 girls.

The mean value of Primer Melting Temperature (Tm) was 78.93 ± 0.8. The concentration in nanograms was determined, and considering the chain length, the number of copies/ng was obtained for the solution and then adjusted to 1 X 10¹¹ and then serial dilutions were made until extinction. Using the standard curve, the detection limit was determined to be between 10²-10³, the last value was chosen to increase the margin of certainty of the test. The standard curve has a 0.0165 error and efficiency of 1.559 (Fig. 1).

The amplification using as initiator the gene spaP of the nucleic acids extracted from S. mutans resulted positive for 91.3% of the cases. Every child with caries was positive for the spaP gene and only 8.75% were negative, including children without caries. The levels of S. mutans in the dental plaque samples were determined by the Threshold Cycle (Ct), which reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. The spaP of S. mutans was detected in 85.5% of the group without dental caries (n=42) and 10% of the groups with patients with childhood caries (Fig. 2). During the amplification product characterisation, the graph presented only one high point Tm at 78.63 ± 0.1221°C; thus, the determinations were specific and there was no presence of initiator dimers or other unspecific amplifications (Fig. 3). The dental plaque sample results were correlated with Early Childhood Caries. The gene spaP of S. mutans detection in bacterial plaque was highly significant (r=0.85, p<0.01) in relation with average values of caries index (Table 5).

The S. mutans recount in relation to patient’s age presented a proportional increase in the number of copies from the 12-18 months range up to 25-32 months. Then, it descended between 33-39 months and increased again in the last age range. The average age of the patients that had a positive spaP was 24 months, while those with negative spaP were in average 30.74 months.

The age of the patients was correlated with gene spaP

**TABLE 2** - The amplification programme used to generate the standard curves for absolute quantification of spaP for S. mutans gene.

**TABLE 3** - Age group and gender frequency.

**TABLE 4** - Boys frequency according to dental caries diagnosis and presence of S. mutans spaP gene.

![FIG. 1 - Standard curve for chain reaction test of polymerase for bacterial quantification constructed using serial dilution of amplicones produced during the reaction. The horizontal axis shows the number of cycles when the fluorescence has reached the limit. The vertical axis indicates the number of S. mutans cells.](image-url)
acquisition using the Spearman's correlation, given that data distribution was not normal, finding a strong correlation between the two variables (r=0.428, p<0.01). Patient's age was also correlated with the caries index (dmft), the results showed that as age increases, the dmft increases too. Both variables were highly correlated (r=0.375, p<0.01).

Discussion

A reliable technique for detecting the presence of S. mutans at an early stage as well as its distribution ratio, is important in order to predict caries risk and thus the treatment.

In the present study the measurement of the Streptococcus mutans was performed through the analysis of bacterial plaque; many authors have stated that the analysis of bacterial plaque is better and more reliable than measurements of SMG in saliva [Alaluusua and Renkonen, 1983]. However, other authors have demonstrated that the results obtained in saliva tests are comparable to those obtained with direct cultures of bacterial plaque [Mundorff et al., 1990].

To strengthen the hypothesis that samples of bacterial plaque are better than those made of saliva, one must observe that saliva's bacteria have their origin not only in plaque but in different oral sites, and that plaque composition varies throughout the day. There are numerous microbial studies from dental plaque for the determination of cariogenic bacteria in the buccal cavity [Hata et al., 2006; Costa and Franco, 2007] and even when the controversy between the two ideas remains, the correlation between bacterial levels and dental plaque has been demonstrated.

This investigation was based on dental plaque samples with tested validity on epidemiology tests and its simplicity to collect samples, translated in a suitable technique [Mundorff et al., 1990].

Oho et al. [2000] determined the presence of S. mutans and S. sobrinus in human saliva detected through conventional PCR. In this study the correlation between the percentage of Streptococcus mutans (gen spaP) found in the dental plaque detected by real-time PCR and dental caries status was analysed.

The qPCR technique was selected because of its precision and reliability. A wide range of bacterial cells can be measured and the specificity of PCR detection can be provided by specific sequential amplification primers which are equally specific for the blank species [Choi, 2009]. The gene spaP was used to design PCR primers because of the great ability to express important virulence factors of S. mutans.

The real-time system allowed initial concentration quantification of the nucleic acid present in the samples on a simpler basis, precise and with a larger range (5-6 log) than conventional procedures (2-4 log).

This investigation established a detection system for S. mutans strains with cariogenic potential using the reaction chain of polymerase from dentobacterial plaque samples. Using this technique it was possible to determine S. mutans using genetic markers such as spaP gene that decodes Ag I/II.

The detection of S. mutans by PCR using the spaP gene is more sensitive than using gen gtfB and ftf probes; the sensitivity of this last method is 104 UFC less. In addition, this method is more convenient in routine use than those that use hybridisation for rapid precise diagnosis of S. mutans infections [Ono et al., 1994].

Hajishengallis [1996] proved that Ag I/II has an important role in the initial implantation of S. mutans to the tooth's surface since it produces an immune protective

### Table 5: Correlation between the presence of S. mutans spaP gene and caries index in children.

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Values correspond to frequencies and averages
r = Spearman correlation coefficient (r = 0.825, p < 0.01: high correlation ** Sig=.825).
response when administered orally with A2/N subunit of cholera toxin. This statement agrees with the previously reported data because the presence of considerable amount of positive samples for spaP is related to the development of carious lesions or absence of injuries [Aguilera, 2002]. This corresponds with colonisation characteristics and tooth damage because as the injury damages tissue, the microbial flora changes, allowing for bacterial implantation with higher resistance to acid environment and less oxygen requirements.

The presence of bacterial strains in dentobacterial plaque that have the AG I/II gene enhance the presence of caries because it is possible for S. mutans to adhere to the tooth by the interaction of Ag I/II with proteins in teeth.

The predictive value of S. mutans presence for caries risk is still discussed; in studies involving small children, probably because of the multifactor causes of caries, this indicator is questioned by some authors [Sullivan, 1996] and defended by many others [Sullivan, 1996; Mattos-Graner, 2001; Thibodeau, 1995]. Some studies point out that the increase of colony-forming unit (CFU) may predict mayor caries risk than the quantified value itself [Weinberger and Wright, 1990]. This study showed that children with caries had a S. mutans proportion significantly higher in their dental plaque samples as well as a significant correlation between the patients with caries and gen spaP. These results agree with those of Hata et al. [2006] and Choi et al. [2009] who reported that patients with dental caries had higher S. mutans count.

The prevalence of early childhood caries varies: 11.7% in Sweden, 62.6% in Puerto Rico and 16.7% in Brazil. The caries prevalence in this study as well as the dmft index was 38.75% and 3.71 respectively, strengthening the results of other studies [Santos, 2002; Cerqueira, 1999; Grøndefjord, 1993]. Vachiraropis plan et al. [2004] found a higher prevalence of early childhood caries than ours (82.8%) in a sample of 520 children 6-19 months old. Rosenblatt et al. [2002] found a 28.46% early childhood caries prevalence when they studied 468 children, a lower value than the one found in this study. In any case, the differences found in the studies may be due to real variation in the colonisation levels and diet habits between different populations that allow the development of dental caries, among other factors [Kalbeek and Verrips, 1994].

**Conclusion**

Based on the observations made at the end of this study, we conclude the following:

- There is a correlation between the presence of gen spaP of S. mutans and early childhood caries patients.
- There is a close correlation between early childhood caries prevalence and the percentage of S. mutans detected by real-time PCR.
- There was a greater prevalence of early childhood caries in female patients and in patients with age range of 40-46 months.
- The spaP gene was present in all patients with early childhood caries and in a larger proportion in patients without caries.
- S. mutans can be detected quantitatively and sensitively by means of real-time PCR with gen spaP initiators.
- Using this method, it is possible to obtain information about the quantity and quality of infection potential of S. mutans and help planning oral intervention programmes.

**References**


