Distribution of *Streptococcus mutans* and *Streptococcus sobrinus* in Saliva of Mexican Preschool Caries-free and Caries-active Children by Microbial and Molecular (PCR) Assays

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The aim of the study was to investigate the distribution of mutans streptococci (MS) infection of caries-free (CF) and caries-active (CA) preschool Mexican children by microbial and molecular assays. Eighty preschool children were divided into two groups, 40 CF and 40 CA children. Saliva samples were inoculated onto MSB to identify CFU and DNA extractions were tested by PCR. Our results indicated that there was no statistical difference ($p>0.05$) between groups either in age, weight, height or sex. S. sobrinus was detected by PCR twice as much in the CA group, the difference being statistically significant ($p<0.05$). dmfs index was positive correlated with S. mutans ($r=0.2941$, $p=0.0081$), S. sobrinus ($r=0.3384$, $p=0.0021$) and S. mutans-S. sobrinus ($r=0.3978$, $p=0.0003$). ANCOVA revealed that dmfs index had a significant effect on the distribution of CFU of S. mutans ($p=0.0118$) and S. sobrinus ($p=0.03$). When MSB was compared with PCR to identify MS, there was no statistical difference ($p>0.05$). We conclude that S. mutans and S. sobrinus were isolated in higher numbers from CA children and those harbouring both bacteria had higher dmfs scores. PCR is a useful tool in molecular epidemiology for dental caries studies; it was effective in detecting and identifying MS from saliva in children.

Keywords: *Dental Caries; PCR; Streptococcus mutans; Streptococcus sobrinus; Saliva; Mexico*
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INTRODUCTION

In spite of all efforts to decrease the incidence and prevalence of dental caries around the world, there are effective results only in industrialized countries where the population has access to primary dental care from school facilities by the application of fissure sealants and fluoride supplements.[1] The opposite scenario is found in undeveloped countries where dental caries still is a leading oral health problem; even with the effort to reduce dental caries through fluoride supplements (drinking water, tooth paste, salt, soft drinks, etc.), poor children from Latin American countries are particularly at risk, as two-thirds of this population have untreated tooth decay.[2-4]

Mutans streptococci (MS) group has been implicated as primary microorganisms, which cause dental caries in humans and experimental animal models, this finding relying on sampling to isolate colony-forming-units (CFU) from dental plaque and/or saliva. Two species of MS group have been associated with dental caries in humans, *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*), these species often being isolated from saliva of patients affected by dental caries.

S. mutans and *S. sobrinus* have been found in animal models. *S. mutans* is associated with pit and fissure caries, while *S. sobrinus* with smooth surface caries; therefore, it is

important to identify both species in clinical trials[5]. Some epidemiological studies have suggested that the presence of *S. sobrinus* is closely related to severity of dental caries rather than isolating *S. mutans* by itself.[6,7]

Mitis-Salivarius-Bacitracin agar plates (MSB) has been the traditional method used for epidemiological studies related to isolation, identification and quantification of *MS* bacteria[8]. However, recently there have been reports of a molecular biology approaches for epidemiological studies. These techniques provide an insight into the era of molecular epidemiology for dental caries studies.[9,10] Since *MS* produce glucosyltransferase (GTF) enzymes that use sucrose as a substrate to synthesize extracellular polysaccharide, which is a main factor in adherence to tooth surfaces; therefore, *gtf* gene is used as target gene to identify *S. mutans* and *S. sobrinus*.[10]

Whole saliva is a complex mixture, formed primarily from salivary gland secretions, gingival fluid, epithelial cells, bacteria, leucocytes and food residues. Saliva is essential for maintenance of healthy oral tissues, as it coats the oral mucosa and protects against irritation, forms an ion reservoir for tooth remineralization, aids in swallowing and has an antimicrobial action.[11] Recently, saliva samples have been considered as an important diagnostic tool to detect risk patients for several diseases.[12] Salivary *MS* counts rarely exceed 10^7 CFU/ml and a highly significant correlation has been demonstrated between the salivary numbers of *MS* and caries prevalence; most studies of this bacterium and its role in dental caries having been carried out in Europe and Japan.[6,13-15] Information regarding the genetic groups of *MS* associated with dental decay in children is important in understanding and evaluating the trends of dental caries in cross-populations studies. The aim of this study was to investigate the distribution of *MS* infection in saliva of a sample of preschool Mexican children with and without dental caries by MSB agar plates and PCR assays.

MATERIAL AND METHODS

Patients

This cross-sectional study involved 80 (46 males and 34 females) children with primary dentition, all of them residents in the city of San Luis Potosi (north-central), Mexico. The Department of Pediatric Dentistry of the Advanced General Dentistry Program undertook Child recruitment. Parents of children completed a standardized health questionnaire that included information about pediatric and oral evaluations, and the last course of antibiotics. An informed and voluntary written consent from parents was obtained prior to clinical examination according to the ethical guidelines of the Helsinki Declaration (1975), and the protocol was approved by the Master's Degree in Dental Science with Specialization in Advanced General Dentistry Program at San Luis Potosi University, Mexico. A non-probabilistic consecutive sampling was performed. This pediatric sample was divided into two groups of 40 children each: A) Caries-free (CF), without clinical presence of dental caries and restorations B) Caries-active (CA) affected by dental caries without restorations. Inclusion criteria were selected in children from 3 to 6 years from either gender. Exclusion criteria included children who had received antibiotics during the last three months. The outcome variable was isolation and quantification by MSB and identification by PCR of *S. mutans* and *S. sobrinus*. The explanatory variable studied was: dental caries (*dmfs* index).

Dental caries

The WHO caries diagnostic criteria were used for determining the *dmfs* (decayed, missing and filled teeth of primary dentition) index.[16]

Saliva sampling

Paraffin-stimulated whole saliva from children was sampled over a 5-min period in a sterilized propylene tube; this process was carried out consistently in the morning (9-10 am) to minimize the circadian rhythm effects, 2 h after the previous meal. During the clinical evaluation for dental caries and saliva collection the children were comfortably seated in a ventilated and lighted room along with their mothers. Saliva samples were stored at -40°C until PCR evaluations were performed.

Microbiological Analysis

Fresh saliva samples were dispersed by sonication for 10 seconds and 10 fold dilutions were prepared in saline solution (0.9% NaCl), 100 µl of each dilution was spread by spiral bacterial plater (in duplicate) onto MSB supplemented with 20% of sucrose and 1% of potassium tellurite. The plates were incubated in an atmosphere of 10% CO_2 at 37°C for 48 hours(h) ; then plates were incubated for 24 h at room temperature. Thereafter, a stereoscopic microscope (Olympus, SD-ILK, Japan) was used to verify the presence of CFU resembling *S. mutans* and *S. sobrinus* morphology and results were described as CFU/ml.[17,18]

DNA extraction and PCR

Bacterial cells from 750 µl of each saliva sample were concentrated by centrifugation (13 000 rpm for 10 min) into a micro-centrifuge tube, washed in 1 ml of PBS (pH 7.4), resuspended into 200 µl of cell lysis buffer (1.0% Triton X-100, 20 mM Tris-HCl, 2 mM EDTA (pH 8.0) and incubated at 85°C for 10 min.[19] Then 100 µl of 200 Units/ml of mutanolysin (Sigma, St. Louis, MO, USA) were added and incubated at 50°C for 1 h, followed by treatment with 80 µl of nuclei cell lysis solution at 80°C for 10 min. Then, 60 µl of protein precipitation solution was added; proteins were then removed by centrifugation (13 000 rpm for 10 min). The DNA was purified by phenol-chloroform-isoamyl alcohol (25:24:1, v/v; Invitrogen, Carlsbad, CA, USA) extraction and isopropanol precipitation. The extracted DNA was dissolved in 100 µl of DNA hydration solution; this solution was used for PCR assay. All reagents were used according to the manufacturers protocol (Puregene DNA isolation Kit, Gentra Systems, Minneapolis, MN, USA).

For further identification of *S. mutans* and *S. sobrinus,* 4 colonies on MSB from each patient were randomly selected from a well-spread plate, and then were grown in 5 ml of BHI (Brain Heart Infusion Broth, Difco Laboratories, Detroit, MI, USA) to an optical density of 1.0 at 550 nm. Bacterial DNA extraction was carried out with the same protocol described above. PCR assay, for both saliva and colonies isolated from MSB agar plates, was carried out in 25 µl of a reaction mixture containing 0.5 U Taq polymerase (Roche), 0.5 µM of specific primers, 5 µl (10 ng/µl of DNA template) and 1.5 mM of $MgCl_2$, following the manufacturers protocol. The PCR reaction was performed in a thermal cycler (iCycler, BIO-RAD laboratories, Hercules, CA, USA) with the following cycling parameters: an initial denaturation at 98°C for 3 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 70°C for 1 min, extension at 70°C for 1 min and a final extension at 70°C for 4 min. Positive and negative controls were included in each PCR set by using DNA from strains (GS5, MT8148 and OMZ 175) for *S. mutans* and strains (6715, B13 and ATCC 27351) for *S. sobrinus*. The species-specific primers for *S. mutans* (MKD) and *S. sobrinus* (MKT) were as follows; MKD-F: 5'-GGC ACC ACA ACA TTG GGA AGC TCA GTT-3', MKD-R: 5'-GGA ATG GCC GCT AAG TCA ACA GGA T-3' and MKT-F: 5'-GAT GAT TTG GCT CAG GAT CAA TCC TC-3', MKT-R: 5'-ACT GAG CCA GTA GTA GAC TTG GCA ACT-3', a 433 bp band and 328 bp band were obtained for *S. mutans and S. sobrinus*, respectively.[10] The PCR products were analyzed by electrophoresis in a 2% agarose gel using Tris-acetate-EDTA buffer, the 100-bp DNA ladder marker (New England Biolab, Beverly, MA, USA) being used as molecular size. Each gel was stained with ethidium bromide (0.5µg/ml) and photographed under UV illumination (Chemi Doc, BIO-RAD laboratories, Hercules, CA, USA) for final analyses.

Statistical Analysis

Before starting the study, two examiners were calibrated in all variables with an expert in pediatric dentistry and a microbiologist, through Kappa test. All variables included were analyzed. All data are expressed as mean ± standard deviation and range. Shapiro-Wilks, Levene and Brown Forsythe tests were used to test the distribution of variables. The non-parametric Mann Whitney U test was used to compare continuous variables; X^2 of Mantel-Haenszel test was used to compare categorical variables. Analyses of covariance (ANCOVA) were used to examine the relationship between CFU of MS (*S. mutans* and *S. sobrinus*) and dental caries, controlling for flow rate, pH and buffer capacity of saliva. The correlation between variables was evaluated by Spearman´s Rho test. JMP program version 5.1 and Stat View (both programs for SAS Institute, USA) were used for statistical analysis, statistical significance was set at $p< 0.05$.

RESULTS

The inter-observer reproducibility regarding dental caries diagnostic reached by two examiners showed a Kappa of 1.0.

Comparison of morphometric traits

The mean of the morphometric information for the children included in the CF group were as follows: age of 4 years and 6 months old (24 males and 16 females), weight of 18.36 kg and 1.05 m of height. In the CA group these were as follows: 4 years and 4 months old (22 males and 18 females), weight of 17.07 kg and 1.05 m of height. In the following traits: age, sex, weight and height, there was no statistical difference ($p>0.05$) between groups. The homogeneity of the different variables included in this study allowed us to compare with confidence the children in relation to the presence or absence of dental caries.

CFU of *S. mutans* and *S. sobrinus* from MSB agar plates

The means of CFU of *S. mutans* in both groups studied had a difference in order of magnitude in CF children as compared to that in CA children, $7.4x10^4$ and $4.3x10^5$, respectively. There was a significant statistical difference ($p<0.05$) using Mann-Whitney U test between groups. The CFU of *S. sobrinus* was isolated in an order of magnitude lower in CF children than in CA children ($7.5x10^3$ and $6.5x10^4$ respectively), this being a significant statistical difference ($p<0.05$).

Frequency of isolation of *S. mutans* and *S. sobrinus* from MSB agar plates confirmed by PCR (Table 1)

The percentage of *S. mutans* isolation was 60 for CF children and 75 in CA children; there was no statistical difference ($p>0.05$) for this specie. However, *S. sobrinus* was

Table 1. Frequencies of identification of S. mutans and/or S. sobrinus from MSB confirmed by PCR.

Variables		Caries-free Group		Caries-active Group		*p
		Frequency	%	Frequency	%	
S. mutans	Positive	24	60.0	30	75.0	0.0510
	Negative	16	40.0	10	25.0	
S. sobrinus	Positive	9	22.5	20	50.0	0.0333
	Negative	31	77.5	20	50.0	
S. mutans &	Positive	7	17.5	17	42.5	0.0119
S. sobrinus	Negative	14	35.0	7	17.5	

40 children for each group; positive: presence of *Streptococcus mutans* and/or *Streptococcus sobrinus*; *X2 Mantel-Haenzel; MSB: mitis-salivarius-bacitracin agar plates; PCR: polymerase chain reaction

isolated twice as much in presence of dental caries, 22.5% in CF children and 50% in CA children. In this case, there was a significant statistical difference ($p<0.05$). The percentage of isolation of both *S. mutans* and *S. sobrinus* was 17.5 for CF children and 42.5 in CA children; there was a significant statistical difference ($p<0.05$).

Frequency of identification of *S. mutans* and *S. sobrinus* from saliva by PCR (Table 2)

The identification patterns by PCR and CFU isolation *of S. mutans* and *S. sobrinus* from MSB were similar; the percentage of *S. mutans* identification from saliva was 55 for CF children and 75 in CA children. There was no statistical difference ($p>0.05$). However, *S. sobrinus* was detected more than twice as much in presence of dental caries, 52.5% in CA children and 20.0% in CF children, this having been a significant statistical difference ($p<0.05$). The identification of both genetic groups, *S. mutans* and *S. sobrinus* was twice as much in the CA children as in CF children, 16 (40%) and 8 (20%), respectively; there was a significant statistical difference ($p>0.05$). When MSB method was compared to detect MS from saliva with PCR technique, there was no statistical difference ($p<0.05$).

Correlation of *S. mutans* and *S. sobrinus* and *dmfs* index in both groups (Table 3)

The mean of *dmfs* index for the CF group was 0. The mean of *dmfs* for the CA group was 4.14 ± 4.8 (range of 1-15) and was positively correlated with the isolation of *S. mutans* (n=80, r=0.2941, $p=0.0081$), *S. sobrinus* (n=80, r=0.3384, $p=0.0021$) and both *S.mutans/S. sobrinus* (n=80, r=0.3978, $p=0.0003$). These findings were confirmed by ANCOVA test, which revealed that dental caries had a significant effect on the distribution of CFU of *S. mutans* ($p=0.0118$) and *S. sobrinus* ($p=0.03$).

Isolation of *S. mutans* and *S. sobrinus* and levels of caries experience (Table 4)

The relationship between isolation of *MS* measured by CFU and different levels of caries experience (CA group) showed that *S. mutans* was found in the same order of magnitude in all levels of dental caries. However, *S. sobrinus* increased its frequency of isolation in close relation with the higher levels of caries activity (*dmsf*) in each category studied. The total CFU (*S. sobrinus* and *S. mutans*) increased according with the levels of caries experience (1.5×10^5, 2.0×10^5 and 4.1×10^5, respectively).

DISCUSSION

The most important risk factor in any disease is the causal agent; for dental caries there are two microorganisms involved: *S. mutans* and *S. sobrinus*.[20] In this study, an important point to note was the percentage of *S. mutans* isolation, which was similar in CA and CF children; these findings providing further support that *S. mutans* is involved in the initiation of dental decay in early childhood.[21] On the other hand, the number CFU of *S. sobrinus* isolated was more than twice as high in CA children as in those CF caries, this result suggest that *S. sobrinus* was associated with active dental caries and children with caries experience. These findings agree with studies carried out in preschool children where there was reported a close association between *S. sobrinus* and dental caries in smooth surfaces.[6] Due to these findings, it is possible that future preventive targets will be

Table 2. Frequencies of identification of S. mutans and/or S. sobrinus from saliva by PCR

Variables		Caries-free Group		Caries-active Group		*p
		Frequency	%	Frequency	%	
S. mutans	Positive	22	55.0	30	75.0	0.0608
	Negative	18	45.0	10	25.0	
S. sobrinus	Positive	8	20.0	21	52.5	0.0025
	Negative	32	80.0	19	47.5	
S. mutans &	Positive	8	20.0	16	40.0	0.0020
S. sobrinus	Negative	17	42.5	5	12.5	

40 children for each group; positive: presence of *Streptococcus mutans* and/or *Streptococcus sobrinus;* *X2 Mantel-Haenzel; PCR: polymerase chain reaction.

Table 3. Correlation of CFU of S. mutans and S. sobrinus with the dmfs index in both groups

Variables	Mean	SD	Range	*p
dmfs index	4.1	4.8	0 - 15	
S. mutans	2.5 x 105	5.4 x 105	0 - 3.1 x 106	0.0081
S. sobrinus	3.6 x 104	1.5 x 105	0 – 1.1 x 106	0.0021
S. mutans & S. sobrinus	2.8 x 105	5.9 x 105	0 – 3.1 x 106	0.000

CFU: colony forming units; SD: standard deviation; *S. mutans: Streptococcus mutans; S. sobrinus: Streptococcus sobrinus*; *Spearman Rho test; n=80. Results are expressed as CFU/milliliter; dmfs: decayed, missing and filled teeth of primary dentition.

Table 4. Salivary counts of S. mutans and S. sobrinus in relation to dmfs index for the caries-active group.

dmfs index	n	%	*S. mutans*	*S. sobrinus*	*S. mutans* *S. sobrinus*
1-5	9	22.5	5.0 x 104	5.0 x 103	1.5 x 105
6-10	19	47.5	3.1 X 104	6.9 X 103	2.0 x 105
> 10	12	30.0	5.6 X 104	1.0 X 104	4.1 x 105

CFU: colony forming units; *S. mutans: Streptococcus mutans; S. sobrinus: Streptococcus sobrinus;* n=40; results are expressed as colony forming units/milliliter; dmfs: decayed, missing and filled teeth of primary dentition.

focused on *S. sobrinus* rather than *S. mutans* strains.

The results found for *MS* identification by both MSB and PCR did not show statistical differences; however, there were three samples in the CF group without that were positive in MSB (two *for S. mutans* and 1 for *S. sobrinus*), but negative in PCR. In the CA group, there was a positive sample for PCR but negative in MSB. This suggests that both methods are susceptible to handling procedures and that molecular biology methods are susceptible to circumstances in which biological samples are processed, stored and analyzed. However, both methods had similar results in terms of detection and identification of *MS* ($p>0.05$), which means that PCR is a useful tool in modern molecular epidemiology and requires less time than the microbiological identification protocol.

It is important to consider that different strains of *MS* induce different levels of dental decay in animal models, probably because *S. sobrinus* produces acid more rapidly than *S. mutans*.[22] Therefore, it is important to know the rate of *S. mutans versus S. sobrinus*. In addition there are studies that agree with our findings where there have reported children harbouring both *S. mutans* and *S. sobrinus* showing a high incidence of dental caries.[6,7] Probably, the best way to study any infectious disease, such as dental caries, is to consider the interaction among several bacteria in biofilm systems to provide new information on the pathogenesis of dental caries.[23]

This study showed that there is a positive correlation between caries and the number of CFU, especially when both *S. mutans* and *S. sobrinus* were isolated. In other words, the presence of both genetic groups in the mouths of children produces more aggressive dental decay. This finding was confirmed by ANCOVA test, which revealed that *dmfs* index had a significant effect on the distribution of CFU of *S. mutans* and *S. sobrinus*. These data are in accordance with international reports including different populations where it has been suggested that there is genetic predisposition and that microbial acid production is modulated by the environment.[22-25] Another important finding in this study was the close association between dental caries experience (*dmfs* index) and the increased amount CFU of *S. sobrinus* isolated, suggesting that it is important to know not only the total number of CFU during dental caries process, but also to determine genetic groups, *S. mutans* and/or *S. sobrinus*.[6,22]

Several studies have demonstrated that mothers are the primary source of infection in their offspring, although there are reports that provide evidence that non-familiar transfer can occur when environmental conditions favour colonization.[22,24] Because mothers have intense contact with their infants and the fidelity of transmission is a universal trait whereby mothers transmit their indigenous biota to their offspring, mothers are the main point to deal with in order to stop transmission of *MS* for dental caries prevention.[25] Addressing the cause, rather than the symptoms, of this infectious disease is the main approach for the dentist in the future.[20] Diagnostics should be performed by determining both *S. mutans and S. sobrinus* in high risk infants by using microbiological, monoclonal antibodies or including DNA-based tests to determine the strains and their combination in the mouths of the mothers and their infants.[26] Although CFU microbial method using MSB and monoclonal antibodies can provide valuable information as an early risk factor, molecular biology technique approaches such as use of PCR could be used to detect live or dead bacteria.[10] In addition, the molecular biology techniques can determine not only the source of cariogenic bacteria, but also the potential virulence of each strain acquired or likely to be transferred. Another advantage of the molecular methods is that they are able to detect microorganisms that are difficult to grow in *in vitro* conditions.[9,10]

CONCLUSION

S. mutans and *S. sobrinus* were isolated in higher numbers from children with caries and those harbouring both had higher numbers of dental caries. The *dmfs* index was positive correlated with both *S. mutans* and *S. sobrinus*, but the number of CFU for both *S. mutans* and *S. sobrinus* was significantly higher than of those positively for either *S. mutans* or *S. sobrinus* alone. This finding was confirmed by ANCOVA test, which revealed that *dmfs* index had a significant effect on the distribution of CFU of *S. mutans* and *S. sobrinus*. When MSB method was compared to detect *MS* from saliva with PCR technique, there was no statistical difference ($p<0.05$). PCR is a useful tool in molecular epidemiology for dental caries studies; it was effective in detecting and identifying *S. mutans* and *S. sobrinus* from saliva of children.

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