COMPARISON OF ORAL STREPTOCOCCI BIOFILM IN CARIES-FREE AND CARIES-AFFECTED PRESCHOOL MEXICAN CHILDREN

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ABSTRACT

Interaction of oral streptococci biofilm is the main etiological factor for dental caries. The aim of the study was to compare oral streptococci (OS) distribution in the biofilm of primary dentition from caries-free and caries-affected preschool Mexican children. This cross-sectional study involved 40 caries-free and 40 caries-affected children with primary dentition. Each child was examined using the dmfs index, DNA was extracted from saliva and presence of OS was determined by PCR. Data obtained showed no statistical difference regarding age and gender (P> 0.05). Streptococcus mutans (Smut), Streptococcus sobrinus (Ssob) and their combination showed significant statistical differences between groups (P< 0.05). Smut, Streptococcus sanguinis and Streptococcus gordonii had an inverse relation with dmfs index and Ssob had a direct relation similar to combined with Smut. Smut-Ssob combined with other OS showed statistical differences (P< 0.05). In free-caries group Streptococcus gordonii was more frequently identified than Smut. The ratio Smut/Streptococcus sanguinis could represent a high risk of dental caries development; this ratio was higher in the caries-affected (1.18) than in the caries-free group (0.32). In conclusion, OS play an important role in dental caries predisposition and severity, not only the presence of Smut and Ssob, but also the complexity and distribution of OS in the biofilm.

Key words: Dental Caries, Saliva, Streptococcus mutans.

COMPARACIÓN DE LA COMPOSICIÓN DE LA BIOPELÍCULA DE ESTREPTOCOCOS ORALES EN NIÑOS PREESCOLARES MEXICANOS CON PRESENCIA Y LIBRES DE CARIES DENTAL

RESUMEN

La interacción de los estreptococos orales en la biopelícula es el principal factor etiológico de la caries dental, por lo que objetivo del estudio fue comparar la distribución de los estreptococos orales en la biopelícula de niños preescolares con dentición temporal, afectados por caries y libres de esta enfermedad. Este estudio transversal incluyó 40 niños con caries y 40 niños libres de caries con dentición primaria. Cada sujeto fue examinado usando el índice ceo, y se tomó una muestra de saliva, de la cual se extrajo el DNA y se determinó la presencia de los estreptococos orales por medio de PCR. Comparando los dos grupos no se mostraron diferencias significativas en cuanto a edad y género (P> 0.05). La presencia de Streptococcus mutans (Smut), Streptococcus sobrinus (Ssob) y su combinación mostraron diferencias estadísticas entre grupos (P< 0.05). La presencia de Smut, Streptococcus sanguinis y Streptococcus gordonii mostró una relación inversa con el índice ceo, en contraste Ssob así como la combinación Smut-Ssob observaron una relación directa. Smut-Ssob combinados con otros estreptococos orales, mostraron diferencias estadísticas entre grupos (P< 0.05). En el grupo libre de caries Streptococcus gordonii se identificó con mayor frecuencia que Smut. La proporción Smut/Streptococcus sanguinis podría representar un alto riesgo de desarrollo de caries dental, esta proporción fue mayor en los sujetos afectados por caries dental (1.18) en comparación con el grupo libre de caries (0.32). En conclusión, los estreptococos orales juegan un importante papel en el riesgo de caries dental así como en su severidad, no solo la presencia de Smut y Ssob, sino también la complejidad y distribución de los estreptococos orales en la biopelícula.

Key words: Caries dental, Saliva, Streptococcus mutans.

INTRODUCTION

Dental caries has been one of the most prevalent and expensive diseases of humans. It is a multifactorial disease, so in order to understand the role of different bacterial species, it is important to promote a complete model of caries etiology which should include specific species of oral streptococci[1].

Mutans streptococci (MS) [*Streptococcus mutans* (*Smut*), *Streptococcus sobrinus Ssob*)] have been considered to be the principal cariogenic microor-

ganisms[2]. Together with Mitis streptococci [*Streptococcus mitis*, *Streptococcus sanguinis* (*Ssan*), *Streptococcus oralis* (*Sora*), *Streptococcus gordonii* (*Sgor*)] and Salivarius streptococci [*Streptococcus salivarius* (*Ssal*), *Streptococcus vestibularis*], they are the most important constituents of human oral flora[3]. Although mitis and salivarius groups have a low cariogenicity, their interactions could be important for the establishment and maintenance of pathogenic plaque[4]. It has been reported that interactions between cariogenic bacteria such as *Smut* with other dental plaque microorganisms could modulate its cariogenic potential in a biofilm community[5]. Therefore, detection and identification of oral streptococci is considered to be important to understand biofilm organization and to develop treatment alternatives for dental caries prevention.

Oral Streptococci are early colonizers in neonates but the establishment of *Smut* and *Ssob* occurs after dental eruption. Studies have shown that when pits and fissures in occlusal surfaces are initially colonized by a non-cariogenic bacterial flora, they may confer host protection by physically occupying the space and blocking the colonization by cariogenic microorganisms, preventing the onset of dental caries[6]. Therefore, competition between pioneer colonizing bacteria may determine the dental plaque composition after its organization; for example, *Sgor* competes with *Ssan* to adhere to saliva coated hydroxyapatite[7]. *Ssan* promotes aggregation with other oral bacteria modeling the process of maturation of dental plaque[8]. It can also compete with *Smut, and* both species appear after tooth eruption and show direct antagonism *in situ*[9].

Saliva participates in different functions such as tissue protection, swallowing, dental remineralization and antimicrobial action. Its quantity, pH and buffer capacity play a significant role in caries development[10]. Saliva samples have been a valuable, non-invasive, simple method used for the diagnosis, treatment and prevention of some diseases[11]. Several methods have been used to detect the presence of oral streptococci, such as identification of colony morphologies on Mitis Salivarius Bacitracin (MSB) agar plates from saliva samples, but the classification is slow and difficult. A PCR detection method based on amplification of glucosyltransferase genes (*gtf*) has been reported[12], this approach having been used to detect the oral streptococci from whole saliva samples as a proposal for epidemiological studies. The aim of this study was to use a molecular identification method (PCR) to compare the distribution of oral streptococci from whole saliva samples of caries-free and caries-affected preschool Mexican children, to apply this knowledge in future to prevention strategies against dental caries, such as replacement therapy.

MATERIALS AND METHODS

This cross-sectional prospective study involved 80 healthy and cooperative children with primary dentition, all of them living in San Luis Potosi (northern-central), Mexico. Child recruitment was undertaken from the Department of Pediatric Dentistry of the Advanced General Dentistry Program. Parents of children completed a health questionnaire that included information about pediatric and oral evaluations, and the last course of antibiotics. Informed and voluntary written consent from parents was obtained prior to clinical examination according to the ethical principles of the world medical association declaration of Helsinki (version 2002).

The pediatric sample involved two groups of 40 children each selected by non-probabilistic consecutive sampling: A) without clinical presence of dental caries and restorations (caries-free) and B) affected by dental caries without restorations (caries-affected). Inclusion criteria were: age between 3 to 6 years and either gender. Exclusion criterion was children who had received antibiotics during the last three months. The outcome variable was the identification by PCR of *Smut*, *Ssob, Ssan, Sgor, Ssal* and *Sora.* The explanatory variable studied was dental caries (*dmfs* index).

Dental caries

The World Health Organization (WHO) caries diagnostic criteria were used for determining the *dmfs* (decayed, missing, and filled tooth surfaces of primary teeth) index[13].

Saliva sampling

Paraffin-stimulated whole saliva from children was sampled with the method reported previously[14]. Samples were transported on ice and stored at -40°C until PCR evaluations were performed.

DNA extraction and PCR

DNA from saliva was extracted and quantified as described in a previous report[14]. The presence of *gtf* genes in the extracted DNA was determined using species-specific *gtf* primers[3].

PCR assay was carried out in 25 µl of a reaction mixture with the conditions reported previously[3,14]. Positive and negative controls were included in each PCR set by using DNA of the following bacterial strains: *S. mutans* (ATCC 35665, MT8148 (c) and OMZ 175 (f), *S. sobrinus* (6715 (g), B13 (d), and ATCC 27351), *S. sanguinis* (ATCC 10556, ST3, ST202, B220), *S. gordonii* (ATCC 10558), *S. salivarius* (NCTC 8618, HHT) and *S. oralis* (NCTC 11427, ATCC 10557). The DNA of the abovementioned bacterial strains were kindly donated by Dr. Taku Fujiwara of the Department of Pediatric Dentistry from Nagasaki University, Japan.
The PCR products were analyzed by electrophoresis in 2% agarose gel using Tris-acetate-EDTA buffer, using a 100-bp DNA ladder marker (New England Biolab, Beverly, MA, USA) to estimate the molecular size. Each gel was stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination (Chemi Doc, BIO-RAD laboratories, Hercules, CA, USA).

Statistical analysis

Before starting the study, two examiners were calibrated in all variables with an expert in pediatric dentistry and a microbiologist, through the Kappa test. All variables included were blind-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. JMP version 5.1 and Stat View 4.0 (SAS Institute, Cary, NC, USA) were used for statistical analysis; statistical significance was set at $P< 0.05$.

RESULTS

The inter-observer and intra-observer reproducibility regarding the diagnosis of dental caries reached by two examiners showed a Kappa of 1.0.

Comparison of morphometric traits

The mean of the age of the children in the caries-free group was 4.58 years (22 males and 18 females). The mean age of the children in the caries-affected group was 4.25 years (23 males and 17 females). There was no statistical difference in age and gender ($P>0.05$) between groups. The homogeneity of these variables allows us to compare the groups in relation to the presence or absence of dental caries.

Identification of oral streptococci from saliva by PCR (Table 1)

The OS most frequently detected in the caries-free group were *Ssal* (75%), *Ssan* (62.5), *Sora* (60%); the species least frequently detected was *Ssob* (10%). For the caries-affected group, the OS most frequently detected were *Smut* (80%) and *Ssob* (70%); the species least frequently detected was *Sgor* (40%).
S. mutans was identified in 80% of the caries-affected and only 20% of the caries-free children. The frequency of detection of *Ssob* was only 10% in the caries-free children and 70% in the caries-affected group. There was a significant statistical difference between groups ($P<0.05$) for both bacterial species. For *Ssan*, *Sgor*, *Ssal* and *Sora* there was no significant statistical difference ($P>0.05$) between groups.

Frequency of combinations of different oral streptococci (Table 2)

In the caries-free group there was a mean of 2.62 ±1.42 bacterial species detected and in the caries-affected group the mean was 3.85 ±1.43; there was a significant statistical difference between groups ($P<0.05$).
The positive combination of *Smut* and *Ssob* was found in 4 (10%) of caries-free children and in 22 (55%) of caries-affected children. There was a statistical difference between the groups ($P>0.05$). The combination

Table 1: Identification of oral streptococci from saliva by PCR.

SPECIES		CARIES-FREE		CARIES-AFFECTED		*P VALUE
		FREQUENCY	%	FREQUENCY	%	
S. mutans	Positive	8	20	32	80	0.0001
	Negative	32	80	8	20	
S. sobrinus	Positive	4	10	28	70	0.0001
	Negative	36	90	12	30	
S. sanguinis	Positive	25	62.5	27	67.5	0.6392
	Negative	15	37.5	13	32.5	
S. gordonii	Positive	14	35	16	40	0.6442
	Negative	26	65	24	60	
S. salivarius	Positive	30	75	29	72.5	0.7994
	Negative	10	25	11	27.5	
S. oralis	Positive	24	60	29	72.5	0.2371
	Negative	16	40	11	27.5	

n=40 each group; *Chi square test

of *Smut-Ssob-Ssan; Smut-Ssob-Sgor; Smut-Ssob-Ssal; Smut-Ssob-Sora* showed a significant statistical difference between the groups (P>0.05).

In 7 caries-free children most of the bacterial species were absent, but only *Ssal* was detected, while in 8 children in the caries-affected group, most bacterial species were detected but only *Sgor* was absent (data not shown).

Detection of oral streptococci and their interaction with caries experience (Table 3)

The mean *dmfs* index in the caries-free group was 0, there were no decayed, missing or filled teeth. For the caries-affected group the mean was 7.35 ± 3.38 with a range of 2-15. *Ssob* was present in 100% of patients with a *dmfs* index of >10 and was detected in only 58.3% of children with *dmfs* index 1-5, being the only bacterial strain that increased according to caries experience. The frequency of detection of *Smut* (91.6%, 76.2%, 71%), *Ssan* (75%, 71.4%, 42.8%) and *Sgor* (58.3%, 33.3%, 28.5%) decreased when the three levels of *dmfs* increased.

In patients with a *dmfs* index of 1-5, the microorganism most frequently detected was *Smut* (91.6%). In children with *dmfs* index of 6-10 it was *Sora* (80.9%) and in patients with *dmfs* index >10 it was *Ssob* (100%).

Combination of different oral streptococci and their interaction with caries experience (Table 4)

The combination of *Smut-Ssob-Sgor* was found more frequently in children with a *dmfs* index of 1-5. The combinations of *Smut-Ssob-Ssan*; *Smut-Ssob-Ssal*; *Smut-Ssob-Sora* were found more frequently in children with a *dmfs* index of 6-10 (42.8%, 47% and 46%, respectively). The frequency of detection of the combination *Smut-Ssob* (50%, 52.3%, 71.4%) increased when the *dmfs* index increased.

Table 2: Frequency of combination of different oral streptococci.

SPECIES		CARIES-FREE		CARIES-AFFECTED		*P VALUE
		FREQUENCY	%	FREQUENCY	%	
S. mutans *S. sobrinus*	Positive	8	20	32	80	0.0001
	Negative	32	80	8	20	
S. mutans *S. sobrinus* *S. sanguinis*	Positive	4	10	28	70	0.0001
	Negative	36	90	12	30	
S. mutans *S. sobrinus* *S. gordonii*	Positive	25	62.5	27	67.5	0.0001
	Negative	15	37.5	13	32.5	
S. mutans *S. sobrinus* *S. salivarius*	Positive	14	35	16	40	0.0001
	Negative	26	65	24	60	
S. mutans *S. sobrinus* *S. oralis*	Positive	30	75	29	72.5	0.0001
	Negative	10	25	11	27.5	

*Chi square test; n= 80

Table 3: Frequency of detection of S. mutans, S. sobrinus, S. sanguinis, S. gordonii, S. salivarius, S. oralis and their relation to caries experience.

SPECIES	*dmfs* INDEX		
	1-5 (n=12)	6-10 (n=21)	>10 (n=7)
S. mutans	11 (91.6%)	16 (76.2%)	5 (71%)
S. sobrinus	7 (58.3%)	14 (66.6%)	7 (100%)
S. sanguinis	9 (75%)	15 (71.4%)	3 (42.8%)
S. gordonii	7 (58.3%)	7 (33.3%)	2 (28.5%)
S. salivarius	9 (75%)	16 (76.1%)	4 (57%)
S. oralis	7 (58.3%)	17 (80.9%)	4 (57%)

dmfs index (decayed, missing and filled tooth surfaces)

Table 4: Frequency of combinations of oral streptococci and their relation to caries experience.

Combination	*dmfs* INDEX		
	1-5 (n=12)	6-10 (n=21)	>10 (n=7)
S. mutans - S. sobrinus	6 (50%)	11 (52.3%)	5 (71.4%)
S. mutans - S. sobrinus - S. sanguinis	4 (33.3%)	9 (42.8%)	2 (28%)
S. mutans - S. sobrinus - S. gordonii	4 (33.3%)	4 (19%)	2 (28%)
S. mutans-S. sobrinus-S. salivarius	4 (33.3%)	10 (47%)	2 (28%)
S. mutans-S. sobrinus-S. oralis	3 (25%)	10 (47%)	3 (42%)

dmfs index (decayed, missing and filled tooth surfaces)

DISCUSSION

To date there are few molecular and epidemiological studies where the distribution of oral streptococci has been associated with cariogenicity and biofilm composition in caries-free and caries-affected populations[3]. Such information could lead to a better understanding of the roles of different bacterial species associated with the severity of dental caries.

In this study, the main outcome that showed significant statistical difference between the two groups was the detection of *Smut* and *Ssob.* The species least commonly identified (10%) in the caries-free group was *Ssob*; the opposite scenario occurred in the caries-affected group where *Ssob* was identified in 28 children (70%). In the cases of *Ssan, Sgor, Ssal* and *Sora,* there were no statistical difference between groups, but in combination with *Smut* and *Ssob* they showed statistical differences between groups. The most frequently isolated species in caries-free children were *Ssal, Ssan* and *Sora*. These species could play a significant role in the caries process because they can interact to preserve the biofilm integrity. *Sgor* was identified in 35% of the caries-free group. A recent study reported the absence of *Sgor* in caries-free children, these differences possibly being explained by sample size[3]. Some reports indicate that *Sgor* can attenuate some of the virulence properties of *Smut* and inhibit sucrose-dependent biofilm formation[5]. In this study in the caries-free group, *Sgor* was more frequently isolated than *Smut*, which suggests that *Sgor* can compete with *Smut* and *Ssan* to adhere to enamel surface[7]. Some reports say that *Ssan* may also compete with mutans streptococci for colonization sites on tooth surfaces, and may exhibit direct biochemical antagonism *in situ*. Because the cariogenic potential of *Ssan* is lower than that of *Smut*, several investigators have suggested that the *Smut/Ssan* ratio may serve as an indicator for caries risk[9]. In our study, we observed a *Smut/Ssan* ratio in the caries-free group of 0.32 and in the caries-affected group of 1.18; although there was not a significant difference, it suggests that the caries-affected group presents a higher risk factor. Another study reported similar data but collected plaque samples as opposed to saliva samples as done in our study[4]. The early introduction of *Ssan* into the oral cavity of children, or increasing its relative proportions by artificial means, could affect mutans streptococci colonization because it could be deferred. Delayed colonization of *Smut* has been associated with lower caries scores[15]. It could be a treatment alternative for dental caries in children but has not yet been substantiated. It has been reported that *Sora* produces GTF, it has an agonist effect with *Smut* in caries development, because there is evidence that contributes to the establishment of oral biofilms and plays an important role in the subsequent colonization of mutans streptococci[16]. In our study, *Sora*, was the second species most frequently identified in the caries-affected group. There was no significant statistical difference between the groups in the detection of *Sora*, but this species was one of the two species most frequently isolated in both groups.

Smut has been implicated in pits and fissures caries and seems to participate in the early stages of the caries process while *Ssob* is associated with caries on smooth surfaces and is closely related to the severity of dental caries[2,17,18]. However, in this study, the positive combination of *Smut-Ssob* was associated with the caries-affected group, these data agree with some reports that indicate that people who harbour both species have a higher frequency and severity of caries[18,19]. The combination more frequently identified in both groups was *Smut-Ssob-Sora*.

In the caries-free children, 7 of them were negative to most bacterial species except *Ssal*; therefore, this finding suggests that *Ssal* is the bacterial species least related to dental caries. On the other hand, in 8 caries-affected children, most bacterial species were present but only *Sgor* was absent, which agrees with some reports where it was shown that *Smut* produces antibacterial substances termed mutacins that exhibit different degrees of inhibition against other bacteria, including *Sgor*[20].

The frequency of detection of *Smut* showed an inverse relationship with the *dmfs* index, identified in 91.6% of patients with a low (1-5) *dmfs* index and 70% of patients with a high (>10) *dmfs* index. The same pattern was observed with *Ssan* and *Sgor*. On the other hand, *Ssob* was the only species that presented a direct relationship with the *dmfs* index, it was detected in 100% of patients with severe caries, and only in 58.3% of patients with a low (1-5) *dmfs* index. This outcome suggests that *Smut* participates in the initial and *Ssob* in the advanced stages of dental caries. The combination of *Smut-Ssob* was increased in close correlation with the *dmfs* index.

The importance of *Smut* in the etiology of dental caries has been well documented, but there is growing recognition that the cariogenic potential may be determined by complex interactions in dental plaque biofilm rather than solely the virulence pro-

perties of a single organism. In this study the caries-affected group showed higher number of bacterial species than the caries-free group, supporting the theory that the presence of different species modulates the severity of dental caries[20].

Probably the best way to study dental caries is to consider the interaction among several bacterial species in biofilm to provide new information on its pathogenesis by molecular epidemiology studies. Recently, bacteria associated with dental caries have been seen to play an important role in systemic diseases, since they have been isolated from blood of patients affected by infective endocarditis and cardiovascular diseases[21,22].

CONCLUSIONS

In conclusion, there was a different distribution of oral streptococci between caries-free and caries-affected groups of children. *Ssob* alone and its combinations are closely related to the severity of caries. *Smut* is likely to participate at the beginning of the caries process. *Ssan*, *Sgor*, *Ssal* and *Sora* could interact with *Smut* and *Ssob* in the formation of a cariogenic biofilm because their combination with *Smut* and *Ssob* showed statistical differences between the two groups. The identification of oral streptococci by PCR is a reliable, useful and rapid molecular method that can contribute greatly to our knowledge on the composition of caries biofilms.

ACKNOWLEDGEMENTS

This study was supported by CONACYT Grant Number 33650, UASLP-FAI and CONACYT (student scholarship No.173644). The authors thank the personnel of the Master's Degree in Dental Science with Specialization in Advanced General Dentistry Program at The University of San Luis Potosi, Mexico.

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