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ODONTOLOGIA / DENTISTRY ARTICLES

Genotypic diversity of *Streptococcus mutans* associated with the risk factors for dental caries in children

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ABSTRACT. The association between the genotypic diversity of *Streptococcus mutans* and risk factors for dental caries in children attending an educational program in the public sector was investigated. Twenty-one children (2-7 years old) who presented at least three risk factors were allocated into two groups: caries free (n = 12); with caries activity (n = 9). Initially, 210 isolates of *S. Mutans* were analyzed through AP-PCR and in the second intervention (after 12 months), new evaluation of risk factors and dmf-t index was carried out, followed by statistical analysis of the data (Simple Logistic Regression and Chi-square Test). There was an association between genotype diversity and caries (p = 0.05). It was found that 66.6% of the caries-free children had one genotype, while 77.7% in the group with caries had two or more genotypes. Having two or more genotypes increased by seven times the chance of injury. Genotypic diversity was associated with inadequate eating habits and oral hygiene practices. The dmf-t index of children with two or more genotypes increased from 2.64 to 4.64. These findings suggest that harmful habits of oral hygiene and diet may favor colonization by *S. mutans* and greater genotypic diversity, potentializing the risk of dental caries in the children evaluated.

Keywords: *Streptococcus mutans*; genotype; risk factors; dental caries.

Diversidade genotípica de *Streptococcus mutans* associada aos fatores de risco à cárie dentária em crianças

RESUMO. Avaliou-se a associação entre a diversidade genotípica dos *Streptococcus mutans* (*S. Mutans*) com fatores de risco à cárie dentária em crianças atendidas num programa educativo do setor público. Vinte e uma crianças (2-7 anos) que apresentavam no mínimo três fatores de risco foram alocadas em dois grupos: livre de cárie (n = 12); com atividade de cárie (n = 9). Inicialmente, analisaram-se 210 isolados de *S. Mutans* por meio da AP-PCR e na segunda intervenção (12 meses após), nova avaliação dos fatores de risco e índice ceo-d, seguida por análise estatística dos dados (Regressão Logística Simples e Teste Qui-quadrado). Houve associação entre a diversidade genotípica e cárie (p = 0,05). Constatou-se que 66,6% das crianças livre de cárie, apresentavam um genótipo, já 77,7% no grupo com cárie apresentavam dois ou mais genótipos. Ter dois ou mais genótipos aumentou em sete vezes a chance de lesão. A diversidade genotípica também se associou a hábitos alimentares e práticas de higiene bucal inadequados. O índice ceo-d das crianças com dois ou mais genótipos aumentou de 2,64 para 4,64. Estes achados sugerem que hábitos nocivos de higiene bucal e dieta podem favorecer a colonização por *S. mutans* e maior diversidade genotípica, potencializando o risco de cárie dentária nas crianças avaliadas.

Palavras-chave: *Streptococcus mutans*; genótipo; fatores de risco; cárie dentária.

Introduction

*Streptococcus mutans*is considered the main microorganism in the etiology of dental caries (Holbrook & Magnúsdóttir, 2012); thus, studying it is vital to public health, since the last epidemiological survey data on oral health conditions of the Brazilian population (Brasil, 2011), showed that children under five years old already present up to two experiences with tooth decay, emphasizing that the component 'c' (with dental caries) is responsible for 80% of the dmf-tindex (index of carious deciduous teeth, extracted / with indicated extraction and restored).

S. mutans displays intense genotypic diversity, but the role of this variation is still little understood (Lembo, Longo, Tsuzuki, Rodrigues, & Mayer, 2007). It is believed that in the light of this diversity, the different genotypes have virulence capacities that vary among themselves (Napimoga, Höfling, Klein, Kamiya, & Gonçalves, 2005) and that the ability of

specific genotypes to compete with other strains would be essential for their colonization of the oral cavity (Longo, Mattos-Graner, & Mayer, 2003). Consequently, some genotypes could colonize the host and lead to tooth decay better than others (Lembo et al., 2007).

The investigation of genotypic characteristics of *S. mutans* and the use of molecular biology techniques have allowed a significant development of the understanding of the process of tooth decay (Liu & Li, 2007). The community of *S. mutans* isolated in saliva is diverse, as has been demonstrated by genotyping techniques of PCR with arbitrary primers (AP-PCR). It was also demonstrated that different genotypes might present different cariogenic potential (Damé-Teixeira, Arthur, Parolo, & Maltz, 2014).

Many studies confirm the correlation between genotypic diversity of *S. mutans* and dental caries prevalence (Pieralisi et al., 2010); Gamboa, Chaves, & Valdivieso, 2010; Zhou, Qin, Quin, & Ge, 2011; Jiang, Yu, Min, Chen, & Zhang, 2012; Cheon et al., 2013);however, the relationship of these genotypes with other risk factors for dental caries, to which infant patients are exposed, has been poorly investigated, and little is known about the diversity and virulence of *S. mutans* genotypes isolated in different conditions of the host.

Streptococcus of the group *mutans* have been found in practically all individuals with high, medium and low prevalence of dental caries (Carlsson, Olsson, & Brathall, 1985; Jiang et al., 2012), and they are even present in healthy oral environments, though with lower prevalence (Jiang et al., 2012). This reveals that only the presence of the microorganism does not guarantee the development of the disease, which is of multifactorial character. In addition to intrinsic factors of the host, behavioral factors such as the duration and frequency of feeding, using bottles at night and the use of liquids with high sugar and lipid densities (Feldens, Giugliani, Vigo, & Vítolo, 2010) and the social aspect are involved in the development of dental caries in early childhood.

Tabchoury et al. (2011), demonstrated with and *in vivo* study that isolated genotypes in the presence of sucrose presented higher acidogenicity than those isolated in the presence of water, thus suggesting that more aciduric and acidogenic genotypes might be present in the biofilm with higher sucrose concentration, being able to form tenacious biofilms and to produce highly cariogenic bacterial plaques.

In this context, this study aimed to evaluate the association between genotypic diversity of *S. mutans* and some risk factors for dental caries in children attended in a educational-preventive program in the city of Maringá, State of Paraná, Brazil.

Material and methods

Experimental design and eligibility of the study population

The study population was composed of children from 2 to 7 years old treated in the public sector, who attended an educational / preventive program every two months since the first year of life. For sample selection, a detailed analysis was conducted of all the medical records of children since the first year of life, by selecting children with deciduous dentition, who presented at least three of the following risk factors for dental caries: inadequate nutrition habits(night feeding; bottle content including sugar or flour; excessive ingestion of carbohydrates - > 5 times a day);precarious oral hygiene (absence of nocturnal hygiene; presence of visible plaques; hygieneper day - < 3 times, children who do not collaborate with home oral hygiene);and others (deep fissures in molars; family history of caries or active tooth decay).

After careful analysis of all the individual records, 21 children were selected and allocated in two study groups: Group 1 – without dental caries (n = 12) and Group 2 –with dental caries (n = 9).

It is worth noting that the genotypes of the 21 children who composed the sample of this investigation were previous study, and the second stage (12 months after) comprised the application of a semi-structured questionnaire to verify the risk factors associated with dental caries present at the time of data sampling and the new evaluation of the dmf-t index.

This project was submitted to the Research Ethics Committee of the State University of Maringá (UEM) for evaluation, and was accepted under No. 500.095 of 09/12/2013, and for the appreciation of the Secretariat of Health of Maringá – PR, Brazil. The legal guardians of the children were informed about the nature of the study and about the requirement for a permit, in accordance with the Professional Ethics Code and the guidelines contained in the Resolution 196 of October 10, 1996, of the National Health Council, for researches involving human beings. After the explanations regarding the risks and benefits of the procedures, all involved signed an Informed Consent form.

First clinical stage, isolation and identification of *S. mutans* trains

Saliva production was stimulated by chewing a piece of parafilm for one minute (American Can Company, Chicago, USA).While chewing, children were instructed not to swallow the saliva or the parafilm. Saliva sampling was performed with disposable wood spatulas (Theoto, Jundiaí, São Paulo, Brasil), described by Köhler and Bratthall (1979), which consists of introducing about 30 mm of the tip of a 150 x 130 mm wood spatula in the mouth of the child and press it at least 10 times (5 times on each side) on the back of the tongue. To remove the spatula, the children were asked to close their lips, without touch the teeth, to retain the excess saliva on the spatula. Immediately after sampling, each side of the spatula was pressed against the surface of *mitis salivarus* agar added with sucrose, bacitracin and potassium tellurite (AMSB) (Gold, Jordan, & Van Houte, 1973; Torres, Pizzolitto, Ellias, & Ito, 1993), distributed in 67 x 15 mm Rodac® plates (Inlab – Interlab Distribuidora de produtos Científicos Ltda, São Paulo, SP, Brazil). The material was incubated in an oven at 37ºC for 48 hours in anaerobiosis jars. With the aid of a stereoscopic microscope (Carl Zeiss, GR), 10 colonies, suspected of belonging to the *S. mutans* developed in the surface of MSB (*mitis salivarius* bacitracin) agar were removed. Each colony was transferred to a microtube containing BHI (brain heart infusion) broth and were incubated for 24 hours in anaerobiosis jars with candle flames at 37ºC. Isolated bacterial cultures were submitted to DNA extraction.

Simplified extraction of bacterial chromosomal DNA

Bacterial cells grown in BHI medium were centrifuged for approximately 10 minutes at 10,000 rpm and the tubes were shed, discarding the BHI medium and retaining only the cells. The cells were rinsed in 500 uL of TE buffer (Tris-HCl 10 mM; EDTA 1 mM pH 8.0) and centrifuged for 5 minutes. This procedure was performed thrice. The samples were submitted to water bath at 100ºC for 10 minutes for breaking of cell membranes and release of DNA, and were centrifuged for 10 minutes. The supernatant containing DNA was removed and poured into new and sterilized microfuge tubes (Eppendorf, São Paulo, Brazil).

Identification of *S. mutans* through PCR

Isolated *S. mutans* were identified through PCR with primers that outflank the glycosyltransferase gene (GTFB-F and GTFB-R -517 pb) previously described (Oho, Yamashita, Shimazaki, Kushimayama, & Koga, 2000) (Table 1).

Table 1. Specific PCR primers for the *S. mutans* glycosyltransferase gene.

Primer	Sequence (5' to 3')	Size of the amplified product (pb)
GTFB-F	5'- ACT ACACTTTCGGGTGGCTT GG-3'	517 pb
GTFB-R	5'- CAG TAT AAGCGC CAG TTT CATC -3'	

The PCR mixture consisted of a final volume of 10 µl with 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 1 µM of each primer, 1U of Taq DNA polymeraseand 2 µl of DNA solution. The amplification conditions were denaturing at 95ºC for 30s, followed by pairing of primers at 59ºC for 30s and extension at 72ºC for 1 min. This amplification was repeated in 30 cycles. After the amplification, 10 µl of the PCR product were analyzed through agarose gel electrophoresis (1.0%). The gel was stained with Syber-safe (Life Technologies, Carlsbad, CA) and the recently synthesized fragments were visualized under ultra-violet light. The size of the product amplified by PCR was estimated from the electrophoretic migration of the product relative to the molecular marker weight DNA Ladder 100 pb (Life Technologies, Carlsbad, CA).

Genotyping by AP-PCR

The AP-PCR was performed with the primer OPA-13 described by Li and Caufield (1998). The comparative analysis of the gels was performed from their alignment, considering each marker of DNA band sizes as reference point. The DNA bands identified to each sample were used to construct a binary matrix, with the values zero (0 –absence of DNA band to the strain assessed) and one (1 – presence of DNA band). This matrix was analyzed using the Jaccard similarity coefficient, which did not consider the negative similarities, i.e. the absence of product. The value scale of this coefficient varies from zero (0 –total dissimilarity) to one (1 - total similarity among the strains). The genetic similarity coefficients matrix obtained was used to obtain a clustering with the method UPGMA, to build dendrograms using the software NTSYS-PC.

Second clinical stage – 12 months after genotyping *S. mutans*

After 12 months of the evaluation of the genotypic diversity, a second questionnaire was

applied, assessing daily practices of the children, regarding nutrition and oral hygiene habits. The assessed variables related to the risk factors for dental caries were:1- Nutrition habits (time of feeding and composition of milk; food ingested between meals; frequency of sugar consumption; children's appetite; frequency in which the grandparents (parents) offer sweets to the children); 2- Oral hygiene habits (if the children brush their own teeth, time of brushing, if the children cry to brush their teeth, if they use dental floss). In addition, the places were the children spend most of their day were also assessed.

The second survey of the dental caries index (dmf-t) was conducted to identify oral health conditions after 12 months, based on the criteria defined by the World Health Organization [WHO] (1999).

Statistical analysis

The statistical procedures were conducted using the Statistical Package for Social Science – SPSS version 20.0, which included the descriptive statistics of the variables surveyed, presenting their frequency and percentage. The association between dependent variable (experience of dental caries) and the independent ones was tested using logistic regression analysis expressed as gross odds ratio (OR) with 95% confidence interval (CI). To analyze the association between the number of genotypes and the risk factors, the Chi-square Test was applied. The results were considered statistically significant when the value of p was ≤ 0.05.

Results

After analyzing the 210 *S. mutans* isolates sampled in the children's saliva, it was possible to verify that in the group of children without experience of dental caries, 66.67% had only one genotype of *S. mutans* and the children with experience, 77.78% presented two or more genotypes. As demonstrated in Table 2, there is a positive association between dental caries and the number of genotypes (p = 0.050). Children with two or more different genotypes of *S. mutans* in their oral cavity presented a 7 times higher chance of developing dental caries (OR = 7, IC 95% : 0.969-50.567) when compared with children that carried only one genotype of *S. mutans* in their oral cavity.

Through the univariate analysis, it was possible to verify the association between some risk factors for dental caries and the number of S. mutans genotypes that children involved in the study had. When the variables related to nutrition habits were analyzed (Table 3), it was observed that 2 or more genotypes were detected in the group of children who had the habit of taking milk before sleeping (72.7%), when compared to the children without this habit (p = 0.06).There was also a greater number of genotypes in the group that consumed milk with cariogenic content when compared to the group without this habit or who consumed pure milk (p = 0.02). Besides, the habit of consuming food between meals presented significant association with the number of genotypes (p = 0.05). 30% of children without this habit or who consumed only fruits presented only one S. mutans genotype, while the children who presented 2 or more genotypes had the habit of consuming something between meals, whereas 54.5% of them consumed cariogenic food. It is worth emphasizing that there was association between grandparents/parents offering sweets to the children and the number of genotypes (p = 0.03), i.e., children with 2 or more genotypes used to receive sweets from the grandparents/parents (81.8%), against 30% of children with only one genotype.

Table 2. Relationship between the experience of dental caries and demographic variables of children and the number of genotypes of *S. mutans*.

Variables	Dental caries experience				OR†	IC (95%)		p value
	No		Yes					
	N	%	N	%				
Age (years)								
1 to 3	3	25	3	33.33	1	0.03	2.69	0.560
4 and 5	7	58.33	2	22.22	0.28	0.19	20.61	0.274
6 and 7	2	16.67	4	44.44	2			
Gender								
Female	3	25	3	33.33	1			
Male	9	75	6	66.67	1.50	0.22	10.0	0.677
Genotypes								
1	8	66.67	2	22.22	1			
2 or more	4	33.33	7	77.78	7	0.96	50.56	0.050*

Simple logistic regression. † Gross odds ratio $p < 0.05$.

Table 3. Association between the risk factors for dental caries (nutrition habits) and number of genotypes of *S. mutans* in the children of the study.

Nutrition habits	Number of genotypes				*p* value
	1		2 or+		
	N	%	N	%	
Take milk before sleeping					
Yes	3	30	8	72.7	0.06
No	7	70	3	27.3	
Content of the milk					
Pure milk	2	20	0	0	
Milk + Chocolate	1	10	4	36.4	0.02*
Milk + Chocolate + sugar	0	0	4	36.4	
Do not take	7	70	3	27.3	
Food offered between meals					
Fruits	3	30	0	0	
Sweets	2	20	6	54.5	0.05*
Varied	3	30	5	54.5	
Do not eat	2	20	0	0	
Frequency in which grandparents/parents offer sweets					
Do not offer	7	70	2	18.2	
Everyday	1	10	6	54.5	0.03*
Weekends	2	20	3	27.3	

*Qui-square test $p < 0.05$.

Table 4 refers to the analysis of the association between the number of genotypes of *S. mutans* and the risk factors for dental caries related to the children's oral hygiene habits.

Table 4. Association between the risk factors for dental caries (oral hygiene habits) and number of genotypes of *S. mutans*in the children of the study.

Oral Hygiene Habits	Number of genotypes				p value
	1		2 or+		
	N	%	N	%	
The children brush their teeth by themselves?					
Yes	1	10	9	81.8	0.002*
No	9	90	2	18.2	
Are they able to use dental floss?					
Yes	5	50	0	0	0.02*
Sometimes	2	20	3	27.3	
No	3	30	8	72.7	
Do they cry to brush their teeth?					
Yes	2	20	8	72.7	0.02*
No	8	80	3	27.3	
Frequency of oral hygiene					
None	0	0	4	36.4	0.03*
Once a day	2	20	4	36.4	
Twice + a day	8	80	3	27.3	

*Qui-square test $p < 0.05$

There was significant association between the number of genotypes and the fact that the children brushed their teeth by themselves ($p = 0.002$), with a higher proportion of children who brushed by themselves (81.8%) with 2 or + genotypes, compared to those who had this habit and presented only one genotype (10%).The use of dental floss was another important risk factor, since 72.7% of the children that did not use it presented 2 or + genotypes, against 30% of children with one genotype ($p = 0.02$). The behavior of the children while brushing was also associated with the number of genotypes ($p = 0.02$): crying was present for 72.7% of the children with 2 or + genotypes, different from the group with one genotype (20%). Regarding the daily frequency of oral hygiene, it was verified that 80% of the children with one genotype brush their teeth twice or more a day, which is statistically different from the group with 2 or + genotypes ($p = 0.03$).

Based on the findings of the first stage of the research, where it was observed that children with 2 or + genotypes would have a seven times higher chance of developing dental caries than children with one genotype, a comparison was performed between the dmf-t indexes of the beginning of the research and those obtained after 12 months, evaluating the increment of dental caries of these children. Table 5 presents the initial dmf-t mean value of 2.64 for children who presented 2 or + genotypes, which increased to 4.64 after 12 months.

Table 5. Initial and after 12 months dmf-t index of children, according to the number of genotypes.

Number of genotypes	dmf-t (initial)		dmf-t (12 months)	
	Mean	Standard Deviation	Mean	Standard Deviation
1	0.60	1.35-	0.90	1.44
2 or+	2.64	3.20	4.64	3.95

Discussion

In the present study, though the number of colony-forming units was not high, the presence of *S. mutans* was found in all of the investigated samples. There was a positive association between caries experience and genotypic diversity of *S. mutans*, in accordance with the reports of previous studies (Napimoga et al., 2005; Pieralisi et al., 2010; Gamboa et al., 2010.; Zhou et al., 2011; Jiang et al.,

2012; Cheon et al., 2013). The higher proportion of children free of dental caries (66.67) presented only one *S. mutans* genotype, while most of those with dental caries (77.78%) presented 2 or more genotypes. The results revealed that have two or more genotypes can be considered a risk factor for dental caries (OR = 7, IC 95% : 0.969-50.567), i.e., children with two or more genotypes have a 7 times higher chance of developing caries than those with only one genotype.

According to Hao, Xu, Chen, Zhou, Zhang and Qin (2014), the increased frequency of consumption of sweets and eating candies before sleeping are important factors for susceptibility to dental caries. Alaluusua et al. (1996) verified that children who consumed high levels of sucrose presented higher incidence of dental caries than those with lower consumption, also finding a greater genotypic variability of bacteria isolated from children with tooth decay, indicating that a large consumption of sucrose might be related to higher diversity of *S. mutans* in the oral cavity. In accordance with these findings, in this study, though not reaching statistical significance, it was observed that among the children with only one genotype, most (70.0%) did not take milk before sleeping, while among those with 2 or more genotypes, 72.7% did it. The addition of sugar and/or chocolate was associated to a greater number of genotypes. ($p = 0.020$).Another nutrition habit that was also significantly associated with the number of genotypes was the consumption of food between meals ($p = 0.048$), and the number of genotypes was greater among the children who consumed cariogenic food in the intervals between meals, when compared to those without this habit, or who consumed fruits. In addition, the offer of sweets by grandparents/parents was associated with the number of genotypes ($p = 0.03$). Most children (81.8%) with this practice presented 2 or more genotypes, against 70% of children whose grandparents/parents did not offer sweets, and presented only one genotype.

To Feldens et al. (2010), regardless the frequency, high consumption of high sugar content food at 12 months of age is a risk factor for early dental caries in childhood. Napimoga et al. (2005) believes that the existence of multiple bacterial genotypes in the dental plaque might be a consequence of favorable circumstances for the colonization of streptococci of the group *mutans*, where a more adequate environment might support the growth of multiple genotypes more adapted to this cariogenic environment. Other studies affirm that sucrose enables the increase of *S. mutans* in the dental plaque, because from this carbohydrate these microorganisms synthesize glucanes that favor their adherence and accumulation in the dental plaque (Loesche, 1986).

Regarding the inadequate habits of oral hygiene, considered as important etiological factors in the development of dental caries, a strong association between the number of genotypes with the practice of brushing their teeth by themselves was found ($p = 0.002$). 81.8% of the children with this habit had presented 2 or more genotypes, against only 10% of the children who presented only one genotype. Concerning the use of dental floss, it was also a risk factor, since among children who did not use it, 72.8% had two or more genotypes, against 30%of children with one genotype ($p = 0.02$). The behavior of the children while brushing also was associated with the number of genotypes, since 'crying' was present for 72.7% of the children with 2 or more genotypes, different from the group with one genotype (20%), ($p = 0.02$). Considering the daily frequency of oral hygiene, 80% of the children with 1 genotype brushed their teeth twice or more a day, which is statistically different from the group with 2 or more genotypes ($p = 0.03$). If we consider that the higher the genotypic diversity of *S. mutans*, the higher are the chances of developing caries, these results are in consonance with studies that declare that oral hygiene habits are important risk factors for the development of dental caries (Cortelli, Cortelli, Prado, Aquino, & Jorge, 2004; Fontana, et al., 2011; Moimaz et al., 2014). According to Martello, Junqueira, and Leite (2012), the greater the presence of thicker and mature biofilm on the dental surfaces, the higher the number of surfaces that present active dental caries. In the study, among the assessed children, those with thick biofilm had 4.25 ($p < 0.001$) higher chances of developing the disease when compared to those with thin or absent biofilm.

If the children in this study had at least three risk factors for dental caries, and considering that the dmf-t index of children with only one genotype grew from an average of 0.6 to 0.9, and among children with two or more genotypes this value increased from 2.64 to 4.64, it was noted that there was an increase in the development of dental caries in children with greater number of genotypes. Because they present these risk factors, being nutrition or hygiene habits, the time involved in the etiology of dental caries became evident. This is, the acquisition of deleterious habits for a long period of time might result in the development of dental caries in a progressive way, which is even more evident in children with a higher number of genotypes, since, for reasons hitherto unknown, *S.*

mutans tends to present a better development in cariogenic environments (Loesche, 1986; Napimoga et al., 2005).

Although the results described above have pointed to differences in genotypic diversity of *S. mutans* in the studied children and to the association with risk factors for caries, including diet and hygiene, further investigations must be carried out by means of more thorough longitudinal studies of genetic polymorphism, in order to elucidate the role of genetics in the bacteria involved in dental caries.

Conclusion

Children with dental caries presented a higher number of genotypes confirming the association of genotypic diversity with this oral disease. The largest number of genotypes is also associated with the presence of inadequate eating and oral hygiene habits. After 12 months, the greater increase in the number of dental caries was verified among children who had two or more genotypes.

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