



Microorganisms related to early childhood caries in a sample of an oral preventive-educative program: a longitudinal study

Microorganismos relacionados a cárie precoce da infância em uma amostra de um programa preventivo de cárie dentária: um estudo longitudinal.

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ABSTRACT

Objetivo: Premature acquisition of cariogenic microorganisms seems to be related to higher prevalence and activity of caries lesions. The aim of the study was to evaluate the prevalence of *Streptococcus mutans* and *Streptococcus sobrinus* in infants enrolled in a dental preventive program and in their mothers, as well as to assess the influence of bacterial prevalence, diet and oral hygiene in dental caries prevalence. **Material and methods:** After clinical examinations (n = 50), saliva and oral biofilms were collected and stored prior to real-time PCR at 6, 12, 18 and 24 months of age.

Results: No correlation was observed between the presence of cariogenic pathogens and diet or hygiene habits at all ages; however, association increased with number of erupted teeth. Salivary levels of bacteria were lower in children than in their mothers at all ages, and children with carious lesions had high ingestion of sugared food.

Conclusion: As the levels of cariogenic pathogens were low in the patients that were enrolled in a preventive program, we can conclude that control of oral biofilm as eruption of infants' teeth occurs and sugar ingestion should be considered of great importance in preventive dentistry, because the association between them was highly positive.

KEYWORDS

Saliva; Dental caries; Oral hygiene; Cariogenic; *Streptococcus mutans*.

RESUMO

Objetivo: A aquisição precoce de microorganismos cariogênicos está correlacionada a alta prevalência e atividade de lesões de cárie. O objetivo deste estudo foi avaliar a prevalência de *Streptococcus mutans* e *Streptococcus sobrinus* em bebês e pares de mães, matriculados no programa educativo e preventivo da Bebê Clínica da Faculdade de Odontologia de Araçatuba (UNESP), como também foi avaliada influência da prevalência bacteriana, dieta e higiene oral na prevalência de cárie dentária. **Material e métodos:** Após exame clínico (n=50), foram coletados saliva e biofilme e submetidos a real-time PCR aos 6, 12, 18 e 24 meses de idade. **Resultados:** Não foi observada correlação entre a presença de patógenos cariogênicos e hábitos de dieta e higiene em todas as idades avaliadas. Níveis salivares foram mais baixos nas crianças do que em suas mães em todas as idades, e as crianças portadoras de lesões cariosas tinham maior ingestão de alimentos açucarados. **Conclusão:** Uma vez que os níveis de patógenos cariogênicos foram baixos nos pacientes que frequentam um programa preventivo, podemos concluir que o controle de biofilme durante a erupção dentária e da ingestão de açúcares pode ser considerada medida de grande relevância em prevenção odontológica, uma vez que a associação foi altamente positiva.

PALAVRAS-CHAVE

Saliva; Cárie Dentária; Higiene Oral; Cariogenicidade; *Streptococcus mutans*.

INTRODUCTION

Dental caries was considered a multifactorial and infection disease associated to bacterial species, named Mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) [1-5]. Some authors state that it is a diet-mediated disease, specially sucrose ingestion [6]. Dental caries is an international public health challenge, especially among young children. Early childhood caries (ECC) is a serious public health problem in both developing and industrialized countries. ECC can begin early in life, progresses rapidly in those who are at high risk, and often goes untreated. Its consequences can affect the immediate and long-term quality of life of the child's family and can have significant social and economic consequences beyond the immediate family as well [7,8]. Studies suggest that there is a higher pH decrease with sucralose biofilm than breastfeeding milk biofilm, which is the most common diet intake in the age ECC is more prevalent [9]. Premature acquisition of these microorganisms seems to be related to higher prevalence and activity of tooth decay in some populations [10-12]. In this sense, infants harbour *S. mutans* and *S. sobrinus* only after tooth eruption, especially with eruption of deciduous molars [13,14] as these microorganisms have strong affinity to oral biofilm and hydroxiapatite, also for teeth fissures and pits [15]. It has not yet been defined how perinatal events influence the acquisition of *S. mutans*, but it is known that it has an important role in the development of dental caries [16].

Ingestion of sucrose is one of the most important risk factors, which leads to the development of caries lesions [4]. Predisponent factors include prolonged and inadequate breast-feeding [17], or use of nursing bottles containing sugared liquids, chocolate milk, tea and fruit juice [5], associated with lack of oral hygiene, mainly in the first year of life [18]. Dental caries in infants can manifest in an

aggressive form, leading to total destruction of the involved teeth in a short period of time, which, in this age, is called ECC [17,19, 20].

The development of studies in the field of prevention and cariology, and the emergence of dental care clinics for infants, promoted a dramatic reduction in caries prevalence and severity, but not enough to meet challenges in early childhood. Studies between mother-child pairs suggest vertical transmission of *S. mutans* in humans [21]. Children, whose mothers present high salivary levels of *S. mutans*, acquire the bacteria more prematurely and in higher amounts than children whose mothers have low levels of these bacteria [8,21].

The use of molecular biology techniques, as real-time PCR, provides more specificity to laboratory tests for the detection of oral microorganisms, allowing a safer determination and quantification of salivary levels of cariogenic species, which is of great importance in the study of infants' oral microflora and correlation between microorganisms and local conditions [12].

In the scientific literature, the majority of studies involving tooth decay, microorganisms, diet, oral hygiene habits, as well as social and economic conditions, are related to schoolchildren, with only few longitudinal works conducted with individuals in early childhood [16,23] and in developing countries [10,24-27].

Considering all factors involved in the initiation process of development of dental caries in early childhood and its prevalence, this longitudinal study aimed to evaluate the prevalence of cariogenic species in saliva and oral biofilm of children at 6, 12, 18 and 24 months of age submitted to oral treatment in a preventive program, assessing association with diet and oral hygiene habits, presence of teeth, dental caries prevalence and gingival condition.

MATERIAL AND METHODS

The study was conducted in accordance with the Declaration of Helsinki. All procedures were approved by the Research Ethics Committee of the Araçatuba Dental School (Protocol 2006 – 01470). All parents were fully informed on the aims and procedures involved in the research, and provided written, informed consent.

Sample Characterization:

Fifty pairs of mother-child comprised the study population. Babies enrolled were participants of the preventive program from the Infant's Dental Care Service of Araçatuba Dental School (UNESP), who fulfilled the following inclusion criteria: age below 6 months; no erupted teeth; no use of medicines at the time of sample collection; informed and signed consent from parents/caregivers.

Clinical examinations were performed under artificial light with the use of a clinical dental mirror. The clinical condition of erupted teeth (prevalence of caries and gingival conditions). The gingival condition was evaluated according to the WHO criteria and dental caries was visual inspection based on the ICDAS was performed [12]. Dental examination was performed by three examiners, and the reliability in classifying tooth surfaces using ICDAS was rated as good, with inter-examiner Kappa coefficient ranging between 0,75 and 0,85.

At the ages of 12, 18, and 24 months, a food register diary was used to evaluate in the children amount, frequency, and composition of consumed food during a 7-day-period. For this evaluation, the Food Diet Registry was used, in which the quantity, frequency and composition of the diet ingested during the 7-day period, which preceded the collection of the clinical specimens, were recorded. The data for each subject were tabulated indicating their pattern of consumption of cariogenic food (low, moderate or high) [25].

In relation to oral hygiene, as these children were part of a school preventive program,

mothers were guided to use toothbrush and fluoride dentifrice twice a day in small portions since the first tooth is erupted. Oral hygiene was evaluated with simple oral hygiene index.

Microbiological procedure

Saliva samples were obtained from the babies at 6, 12, 18 and 24 months of age, in the morning, by gently swabbing alveolar ridges, mucosa, and the tongue. In addition, biofilm samples were obtained only at 12, 18 and 24 months of age (due to the absence of teeth at 6 months of age). Biofilms were collected using sterile paper points, which were placed in the gingival sulcus. Samples were then transferred to tubes containing MilliQ water for storage. For the mothers, saliva and biofilm samples were obtained, stored and processed exactly as described for children at the same occasions (6, 12, 18 and 24 months).

Quantitative detection of *S. mutans* and *S. sobrinus* by real-time PCR

Mothers' and infants' levels of *S. mutans* and *S. sobrinus* were evaluated using real-time PCR and the TaqMan System for amplification with specific probes for the 2 targeted microorganisms labeled in the 5' direction with 6-carboxyfluorescein (reporter dye), and in the 3' direction with 6-carboxytetramethylrhodamine (quencher dye) [19] (table 2). DNA samples were extracted using a commercial kit, InstaGene Matrix (Bio-Rad Laboratories, CA, USA). DNA concentration in each sample was measured in spectrophotometer (Beckman, Model DU-640). DNA amplification was done by determined dilutions and in PCR equipment (Perkin Elmer, GeneAmp PCR System 2400), with determined cycles and temperatures. The amplification was done in 25 μ l volume with 2,5 μ l of 10 X PCR buffer, 1,25 μ l of MgCl₂ (50 mM), 2,0 μ l of dNTP (10 mM), 0,25 μ l of Taq DNA polymerase (0,5 U), 1,0 μ l of each starter (0,4 μ M), 7 μ l of sterilized Milli-Q ultrapure water and 10 μ l of DNA (ng) (Table 1). Amplification was done in PCR equipment (Perkin Elmer, GeneAmp PCR System 2400) programmed as: 1 cycle of denaturation at 94°C (5 min.); 30-

36 cycles at 94°C (30s-1 min.), annealing temperature according to starter, 72°C (30s-2 min.), and 1 cycle DNA final extension at 72°C (5 min.). The products of PCR amplification were subjected to electrophoresis on agarose 1% gel, 90 V for two hours, and stained with ethidium bromide (0.5 ug / ml) and photographed with UV transilluminator for light with Kodak camera (Electrophoresis Documentation and Analyses System 120)

Statistics

Models of hygiene and diet habits, and relationships between the number of microorganisms and the non-microbiological data were evaluated using Fisher's exact test and Chi-square test. The distributions of cariogenic species in children and mothers were evaluated using ANOVA and Tukey test. A *p* value of < 0.05 was deemed to indicate statistical significance SPSS software version 18.0 (IBM Chicago SPS Inc).

Table 1 - Oligonucleotides used in the amplification of microorganism DNA by real-time PCR.

Microorganism	Oligonucleotide used	Amplicon (pb)	Target Gene
<i>Smutans</i>	5'-GCC TAC AGC TCA GAG ATG CTA TTC T-3'	114	<i>gtfB*</i>
	5'-GCC ATA CAC CAC TCA TGA ATT GA-3'		
	5'-GGA AAT GAC GGT CGC CGT TAT GAA-3' (probe)		
<i>S.sobrinus</i>	5'-TTC AAA GCC AAG ACC AAG CTA GT-3'	88	<i>gtfT*</i>
	5'-CCA GCC TGA GAT TCA GCT TG T-3'		
	5'-CCT GCT CCA GCG ACA AAG GCA GC-3' (probe)		

*Glycosyltransferase genes, associated to the production of extracellular polysaccharides.

RESULTS

Evaluation of gingival condition at 6, 12, 18, and 24 months showed that all children presented with gingival tissues in normal condition. In relation to tooth eruption, no children had erupted teeth at 6 months, but at

the other ages investigated, a wide variation in the number of erupted teeth was observed (Table 2).

In relation to oral hygiene, at 6 months 38 mothers (76.0%) reported that they engaged in habits aimed at maintaining their child's oral hygiene, and 12 (24.0%) reported that they did not. This difference was statistically significant (Chi square test, *p* < 0.001). At 12 months, oral hygiene was maintained once a day in 19 children (42.2%), twice a day in 16 (35.6%), 3 or more times a day in 9 (20.0%), and rarely in 1 child (2.2%). At 18 months, oral hygiene was maintained twice a day in 17 (27.7%) children, three or more times in 10, and once a day in 9. Oral hygiene twice a day was significantly more prevalent than the other groups (Chi square test, *p* = 0.041). At 24 months, all 37 evaluated patients used a toothbrush and fluoride paste to maintain oral hygiene. Oral hygiene practices were performed once, twice or 3 or more times a day in 9, 18 and 10 children, respectively.

Regarding dietary habits, at 6 months 36.0% (18) of the children were being exclusively breastfed, 38.0% (19) were being exclusively bottle fed, and 23% (11) were being fed both ways. These 3 conditions were similar in terms of their distribution within the studied population (Chi square test, *p* = 0.805). At 12 months, according to data from the food diaries, all children presented a moderate pattern of ingestion of sugared food. In 2 children, at 18 and 24 months, changes to a pattern of high consumption were evident. This difference was statistically significant (Chi square test, *p* < 0.001).

As no children had tooth eruption at 6 months of age, the prevalence of dental caries was not evaluated at that initial time-point. At 12 months, none of the 45 patients examined exhibited tooth decay. At 18 months, 2 children (5.4%) presented ICDAS 1 lesions. In relation to the number of evaluated children, the presence of tooth decay at 18 months did not differ from that at 12 months of age (Fisher's exact test, *p* = 0.34). At 24 months, 3 patients (8%)

showed active caries lesions involving tooth enamel (ICDAS score3); 2 of these children were the same subjects that had presented with caries lesions at the previous time-point (ICDAS score 1 increased to 3). No significant difference between the ages of 18 and 24 months were verified (Fisher's exact test, $p = 0.13$).

No significant influence of food ingestion or oral hygiene on the prevalence of *S. mutans* and *S.sobrinus* was verified. Salivary levels of *S.mutans* and *S.sobrinus* were significantly elevated in mothers when babies were 6 months old (ANOVA, $p = 0.021$ and $p = 0.007$, respectively) and 12 months old (ANOVA, $p = 0.032$ and, $p = 0.016$, respectively). At 12 months, the number of erupted teeth was the only factor that affected the prevalence of cariogenic microorganisms, which were higher in children who presented 6 or more erupted teeth (ANOVA, $p = 0.033$) in comparison with children presenting fewer erupted teeth, although salivary levels of microorganisms were evidently not similarly affected (ANOVA, $p = 0.16$). At 18 months, higher bacterial levels were observed in children with caries lesions and a high pattern of sugar consumption (ANOVA, $p = 0.027$). Levels of *S. mutans* and *S. sobrinus* were significantly higher in mothers when babies were 18 months old (ANOVA, $p = 0.022$, and $p = 0.028$, respectively). At 24 months, levels of *S. mutans* and *S.sobrinus* remained low in all caries-free children, but were significantly higher in their mothers (ANOVA, $p = 0.018$, and $p = 0.041$ respectively). Children with caries lesions again presented higher levels of these microorganisms (ANOVA, $p = 0.032$).

Table 2 - Variation in the number of erupted teeth in evaluated children at 12, 18 and 24 months of age.

12 months		18 months		24 months	
N° children (=45)	N°erupted teeth	N° children (n=37)	N°erupted teeth	N° children (n=37)	N°erupted teeth
8	≤2	9	≤10	4	10 to 14
15	3 to 5	28	11 to 20	22	16 to 17
19	6 to 8			11	20
3	9 to 10				

DISCUSSION

During the follow-up from 6 to 24 months of age, the factors evaluated in this study (tooth eruption, gingival condition, oral hygiene, diet, and prevalence of tooth decay) showed patterns that could be considered as normal at the ages investigated. This may have been partially due to the fact that these patients and their parents participated in an educative and preventive program.

Tooth eruption showed variability, and an increase in the number of erupted teeth was associated with an elevation in the number of microorganisms. This finding was also observed by article which reported that 26% of children aged 2 to 6 months tested positive for *S. mutans*[27]. Clinical importance is given to this study, as a higher number of erupted teeth, including deciduous molars, is a risk factor for tooth decay in patients at this age, as they offer pits and fissures for microbiological adhesion, reason by which oral hygiene procedures must be intensified as tooth eruption continues. Besides, cariogenic bacterias need tooth surface to adhere permanently in oral environment.

Gingival condition and oral hygiene remained within parameters considered normal, although infants of the ages investigated are dependent on parents' actions to maintain oral hygiene. Such habits are later implemented in the child's routine [1]. For this reason, maintaining oral hygiene depends on the development of motivation and ability, essential factors in babies' oral hygiene routine [10].

In relation to diet, a normal profile was noted in this study, although it was not ideal, few mothers were breast-feeding when babies were 6 months old. Positive correlations have been reported between milk type and time of bottle use, and higher levels of *S. mutans* in children in the first 3 years of life [27]. In our research, there was no significant influence of diet type on the occurrence of cariogenic microorganisms. However, it should be highlighted that factors such as economic and social level, diet, oral hygiene habits, and age at the first dentist

visit cannot be considered in isolation [28]. Moreover, children who have preventive treatment early tend to belong to families of a higher social and economical level, which can result in increased motivation and positive behavior. Diet evaluation was performed only in the children, not in the mothers or in the families. In the diaries, only cariogenic intake was pointed and classified.

In a longitudinal study, it could be verified that children with 30 days of age can harbor MS, also at 7 months, and also harbor LB. Probably these children that show early colonization were also classified at high caries risk. This fact indicates that oral environment leads to acidogenic bacterial growth in oral biofilm [8]. MS is part of a viable microbial community in active dentine lesions [29,30].

Consumption of sugar influence the prevalence of dental caries in patients with high to moderate salivary levels of *S. mutans*, and caries are not seen in children with the lowest levels [31]. However, some authors have reported a positive association between sugar consumption and the prevalence of tooth decay [6,32]. It is also observed that the consumption of carbohydrates, present in cakes and snacks cause caries. For the colonization of *S. Mutans* the number of erupting teeth, eating habits and food factors are important. As it could be seen in this study. Changes in diet and oral hygiene habits are also influential; as children grow up they become responsible for maintaining their oral hygiene, but some may not be capable of doing so [32].

It is important to emphasize that 92% of children in this study does not develop tooth decay in all periods, and prevalence of cariogenic bacterias was low in these patients, and higher in their mothers.

Streptococcus mutans that is a bacterium that is related to the onset and progression of caries [11,23]. Children whose mothers have high levels of *S. mutans* during the predentate phase but low prevalence in the dentate phase, have a small probability of developing tooth

decay [33]. The last 2 periods assessed in the present study (18 and 24 months of age) are considered in the literature to be those with the greatest elevation in microorganism prevalence [34]. A strong correlation between the presence of cariogenic microorganisms in mothers' and their children's mouths was evident in the current study. With regard to the window of infectivity for *S. mutans*, a rapid increase in these microorganisms has been linked to biological events such as tooth eruption, mainly of deciduous molars, which creates a habitat for biofilm development [1]. In this sense, teeth represent virgin areas open to colonization by *S. mutans* and *S. sobrinus*. Data from the present study show that some of the children presented cariogenic microorganisms prior to tooth eruption, and that at the ages investigated, oral microflora forms rapidly.

It was initially believed that children did not harbor these microorganisms until the time of tooth eruption, as it was thought that they needed hard surfaces for adhesion and colonization [11,21,13]. Data from this work suggest that after a transitory period involving increased numbers of erupted teeth, colonization is established, and in approximately 20% of children, mainly in those with dental caries, these microorganisms could definitely colonize the oral cavity before eruption of deciduous molars.

In this study of the prevalence of *S. mutans* and *S. sobrinus*, an increase in the number of erupted tooth in the oral cavity was a more important predisposing factor than diet or oral hygiene habits. Moreover, children with a cariogenic diet and carious lesions exhibited higher levels of these microorganisms, while an opposite trend was seen for caries-free children which increased with age and tooth eruption.

Prevention of tooth decay should be the mainly objective of a preventive program in this age. Although, once established, clinicians should deal with the challenge of determining more appropriate intervention to stop caries process. As the objective is to reestablish

mineral balance, interventions include positive compliance, dietary counseling and oral hygiene instructions with constant motivation. Besides the use of fluoride dentifrice is considered [19].

Early insertion of children in preventive programs with preventive and educative measures can positively influence *S. mutans* transmission and colonization of mouth, although it is inevitable. Moreover, it is unquestionable that diet and oral hygiene habits are established in the first year of children and early insertion in such programs can contribute for healthy development[12]. Periodic biofilm control promote frequent disorganization as occur in these clinics and can also support caries prevention [35].

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