Time of initial acquisition of mutans streptococci by human infants

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The aim of this study was to detect and monitor the acquisition of mutans streptococci (MS) in healthy Brazilian children. Samples of 4 different sites (saliva, tongue dorsum, dental ridges, and dental plaque, if teeth were present) were collected from 33 edentulous nursery school infants (5.9±1.5 month-old), using sterilized swabs, bi-monthly for 24 months. Saliva samples from the mothers were collected only once. After inoculation, and incubation typical morphotype colonies, were isolated and submitted to amplification by the technique of polymerase chain reaction (PCR) for identification. The PCR method identified 1667 strains as MS. In 29 of the children's samples, the first positive culture for MS occurred at 15.3 ± 4.6 months. At the end of the follow-up period, 77% of the children were classified as colonized and in 33% MS was found as a transient microorganism. A positive correlation was found between the time of MS acquisition by the infant and the number of erupted teeth ($p<0.0001$), and the time of emergence of the first tooth ($p=0.0048$). After 24 months, there were no dental caries, and 77% of children remained caries-free. These results indicate that MS colonization in this sample of low-income pre-school children may begin earlier than suggested by some investigations.
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INTRODUCTION

Mutans streptococci (MS), which include *S. mutans* (Sm) and *S. sobrinus* (Ss), are considered as being among the major dental caries etiologic agents. There is considerable current interest in the initial oral MS colonization in children, since this is related to caries risk in pre-school children,[6,7] and even low MS levels can be associated with Early Childhood Caries (ECC) in very young children.[2] Understanding about the acquisition, colonization and transmission sources and routes of these species may be essential for developing strategies to prevent dental caries.

In spite of the fact that several studies have been made to determine the time of initial MS acquisition, this timing is still controversial. Knowing the age at which these pathogens colonize the oral cavity will help to understand disease development and to devise interceptive measures.[3]

MS seem to have a certain optimum colonization period, especially during a discrete time period, called "window of infectivity", ranging from 19 to 31 months of age, with a median age of 26 months, time of emergence of primary molars.[8] Although most studies suggest that MS require a non-desquamating surface in order to colonize and thrive,[8-12] which is present with deciduous tooth eruption at the age of about 8 months (2 months).[13] There is consistent evidence that MS may be found in pre-dentate mouths,[2,5,14-16] or shortly after the tooth eruption.[3,6,14,17-20]

With regard to the temporal aspects of MS colonization, it is necessary to determine whether the MS window of infectivity will shift in agreement with the characteristics of the studied population.[8,12] In an epidemiological study carried out in a Brazilian low socio-economic level population,[6] the MS colonization time in the sample of children was found to be earlier than the period described by Caufield *et al.*,[8] however, due to the minimum age for inclusion in the study (12 months), the initial time of acquisition could not be established.

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Based on these considerations, the purpose of this study, which was a part of a larger one was: (i) to detect the time of MS acquisition in healthy low economic level Brazilian children; (ii) to monitor MS colonization bi-monthly for 24 months, to confirm that presence was not transient; and (iii) to clarify the MS acquisition time relationship with mothers' characteristics (salivary MS level, and caries experience), and characteristics of children (tooth eruption time, and number of erupted teeth present).

METHODS

The study was done in a nursery school population from seven different public day nursery schools, randomly selected from 21 institutions in Piracicaba (São Paulo State, Brazil). These children are from a low economic level and stay at the school nurseries for 5 days per week, 10 h per day (from 7 A.M. to 5 P.M.), because most of their mothers worked as housemaids. Piracicaba is located in one of the most developed Brazilian Regions and has had fluoride in the water supply at optimal adjusted levels (around 0.7 ppm F) since 1971.

The Ethical Committee in Research at the Faculty of Dentistry of Piracicaba approved this study (protocol number 57/2000). Written informed consent was obtained from the parents.

Thirty-three mother-infant pairs out of approximately 280 pairs were selected for this study, based on the following criteria: a) absence of erupted teeth in the child during the first collection visit; b) absence of over four missing posterior teeth and no more than one missing tooth per quadrant;[21] c) absence of any chronic diseases or daily intake of medicines.[21] The median age of the edentulous children during the first sample was 5.9 ± 1.5 months (mean ± standard deviation [SD]). The mothers' ages averaged 28.6 ± 5.3 years. Seven pairs were lost during the 24 month-study because the children left the nursery school (Table 1).

Table 1. Number of mother infant pairs in relation to the follow-up examinations.

Clinical and microbiological follow-up examinations (months)				
	2	**10**	**12**	**24**
Mother infant pairs (female/male child)	31 (18/13)	30 (17/13)	29 (16/13)	26 (16/10)

Initially, the main examiner went through a calibration process, involving calibration with an experienced examiner, and practical training in clinical procedures applied to 4 mother-infant pairs, who were similar in age to those of the volunteers in the study. These volunteers did not belong to the experimental group.

The clinical examinations were done and salivary samples were collected by one of the authors (FMF). Mothers were examined in a dental chair in the supine position under a standard dental operating light. After a dental prophylaxis, maternal caries experience was assessed according to the WHO criteria[22] using a blunt probe and plain mirror, by the sum of decayed, missing and filled teeth (DMF-T). The mothers received complete restorative care as described by Wright *et al.*[21] before the emergence of first teeth of the infant. They were individually informed about the concepts of oral health, transmission of oral microorganisms, and trained in home-based plaque control. Kits containing 1500-ppm fluoride toothpaste, toothbrushes (to all dwellers of each household), dental floss, and an explicative leaflet, including educative aspects related to dental caries (multifactorial etiology, transmission, and prevention) were given to each mother bi-monthly. The mean ± standard deviation of the DMF-T index in the mothers was 10.4 ± 6.3.

The children were examined at the nursery schools during all the every two-month visits. The guiding parameters were the number of erupted teeth, evidence of tooth decay, and the presence of colony-forming units (CFU) resembling MS.

A tooth was considered to be present (erupted) if any part of its crown was visible in the mouth (WHO). The time of tooth eruption was considered as the child's age at which the first tooth was visually detected during the 2-monthly interval visits.

After tooth eruption, child caries experience was assessed. Dental caries was diagnosed by visual examination only, given the young ages of the subjects and the field-examining conditions. Artificial lighting was used. The children had their teeth brushed. Initial and caries cavities of every tooth were recorded and caries prevalence determined. The number of decayed teeth was scored as *dt*, since no missing or filled teeth were found. Initial caries was defined as a demineralized surface with loss of translucency and caries cavities as the visible break in the enamel surface, pit and fissure discoloration with adjacent opacity, evidence of marginal ridge undermining (coronal dentin exposed) (WHO). Mineralization disturbances were not considered. Explorers were not used in these exams.

Microbiological samples

For every infant bi-monthly samples from different sites, at least one hour after the last food intake, were swabbed with sterile cotton swabs.[9,15,20] Saliva samples were collected by placing a sterile cotton swab sublingually until saturated. The maxillary and mandibular dental ridges and the tongue dorsum of predentate and dentate infants were sampled by passing a sterile cotton swab over the tissue. The plaque samples from dentate children were sampled by passing a sterile cotton swab over the buccogingival border and occlusal surfaces of all teeth present. Each swab was placed in

a sterile vial containing 830 µL of saline solution (0.9% NaCl).

Before starting the restorative care of the mother, whole saliva samples were collected in sterile tubes from each of them, after stimulation, in order to assess the MS levels in saliva. Each mother was asked to chew on a piece of paraffin wax until it attained the soft consistency of chewing gum. They then swallowed any saliva present in the mouth, and chewed on the paraffin using both sides of the mouth for a further 1 minute, while spitting saliva into a sterile glass tube intermittently.

All the samples were placed on ice, and plated within 3 hours. Most samples were processed within 90 minutes of collection.

Bacteriological methods

Samples were dispersed by vortex[5,15] for 60 seconds to disperse bacteria and submitted to 10 fold dilutions in saline solution (0.9% NaCl) through 10^{-4}. Aliquots of 5 µL of each dilution were plated, in duplicate, onto Mitis Salivarius Agar[ϑ] supplemented with 2 IU of bacitracin[K]/ml, 1% potassium tellurite[K], and 15% sucrose[Φ] for MS cultivation (MSB). In addition, the swabs were streaked directly onto MSB.[8] All plates were incubated under anaerobic conditions (37˚C, 10% CO_2) for 48 hrs (Water-Jacked CO_2 Incubators/Cole Parmer Instruments – USA).

Thereafter, a stereoscopic microscope was used to verify the presence of colony-forming units (CFU) resembling MS. They were distinguished by their molar tooth, granular frosted glass-like formation, adherent growth in agar and production of glucan surrounding colonies. If present, up to thirty presumptive colonies of MS from children MSB plates (during all the two-month-interval visits), and up to 15 from mother MSB plates were picked at random and subcultured in 3mL of Brain Heart Infusion broth (BHI) – to obtain pure cultures. The selected colonies were those most representative of the colonial morphological patterns observed on each plate. Pure cultures of each strain were then promptly frozen in glycerol stocks at -70˚C for further analysis.

Each isolate was then confirmed by means of Gram stain, catalase activity, and by a method using the Polymerase Chain Reaction – PCR.

Chromosomal DNA was extracted using a simpler DNA preparation in which the cells from a liquid culture (BHI) are washed and simply boiled for 10 minutes with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0), the debris pelleted and the supernatant diluted for identification by PCR (modified from Welsh, McClelland,[28] and Saarela *et al.*[29]).

Oligonucleotide primers were designed according to Oho *et al.* The PCR was processed using 5µL of template solution added to 45µL of reaction mixture containing 1X Reaction Buffer (50 mM KCl[α]), 1.5 mM $MgCl_2$, 200µM each of dATP, dTTP, dGTP and dCTP[α], 1µM of each primer, 1.25 U Taq DNA Polymerase[α]. Beside the samples, purified genomic DNA from *S. mutans* (CCT 3449) and *S. sobrinus* (ATCC 27607) was used as positive control and distilled water was used as negative control. PCR amplification was performed using a GeneAmp PCR System 2400 (Perkin Elmer, Applied Biosystem, Singapore) under the following conditions: 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 59˚C for 30 seconds, an extension at 72˚C for 1 minute, and a final elongation step at 72˚C for 2 minutes.

The PCR products were analyzed by electrophoresis in 1.5% agarose gels[α] using Tris-borate-EDTA buffer (pH 8.0). A 100 bp DNA ladder was included in each gel. The DNA was stained with 0.5 mg/mL ethidium bromide, visualized and photographed under ultraviolet lighting (Pharmacia LKB - MacroVue; Pharmacia Biotech - Image Master -VDS).

The age at which an infant was defined as having been colonized by MS was assigned after two consecutive positive cultures for MS from any sample site. A child was considered non-colonized based upon repeated negative samples for MS taken at two-month intervals for 24 months. If a positive culture for MS was verified in only one period or in non-consecutive occasions (irregular isolation), the presence of MS was considered transient. A child was considered as colonized, based upon two consecutive two-monthly interval visits with positive samples for MS.[8]

Statistical Analyses

Means, standard deviation, standard error, and confidence intervals (95% CI) were determined for all data. The levels of MS in CFU/mL of mother's saliva were converted into logarithm (log_{10}) for the statistical analyses. Spearman correlation was used to verify the relation between the time of initial MS acquisition and: a) the mean age of the first tooth emergence, b) the number of erupted teeth, c) the MS levels of saliva from mothers. The Fisher's Exact test was used to verify: a) the initial MS acquisition sensitivity

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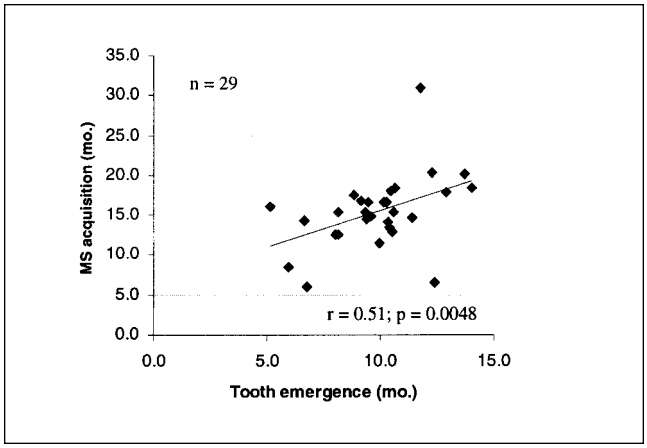

Figure 1. Relationship between the age at first tooth emergence and the time of MS detection. The correlation was statistically significant (r = 0.51; p = 0.0048).

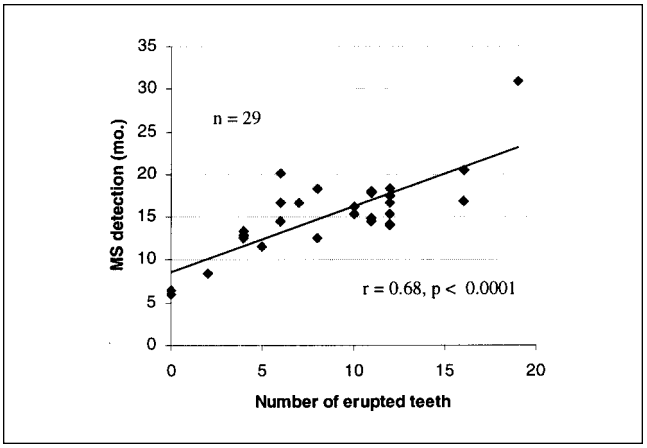

Figure 2. Relationship between the number of erupted teeth and the time of MS detection. The correlation was statistically significant (r = 0.68, p < 0.0001).

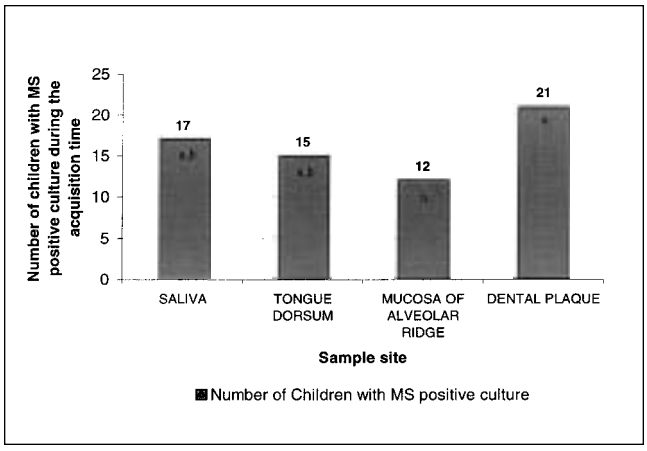

Figure 3. Number of children with MS positive culture during the acquisition time in relation to the sample site. Values followed by different letters in the vertical bars differ among each other according to the Fisher's Exact test (p <0,05).

among the 4 oral sites evaluated, b) the relation between gender and the kind of colonization verified at the end of 24 months (transient or permanent). The One-Sample t-test was used to compare the mean time of initial MS acquisition by the sample child and the time point (window of infectivity) reported in the literature. For all tests, p≤0.05 (two-sided) was considered statistically significant.

RESULTS

MS acquisition in samples from children

Twenty nine of the 33 studied infants showed at least one positive MS culture, at a mean age of 15.3 ± 4.6 months (mean ± SD), ranging from 6.0 to 30.9 months-old. The One-Sample t-test shows that this mean age was prior to the time point for MS implantation reported in the literature (26 months old) (p<0.0001, One-Sample t-test).

The mean age of the first tooth emergence was 9.9 ± 2.1 months. As shown in Figure 1, the time of initial MS acquisition was correlated to the infant's age at first tooth emergence (r = 0.51; p = 0.0048; Spearman correlation analysis).

The number of erupted teeth during acquisition varies from 0 to 19 (8.7 ± 4.7). Two children were edentulous, 12 showed only incisors and 15 had primary molars erupted during this first contact with MS. As shown in Figure 2, the time of initial MS acquisition was significantly correlated to the infant's number of erupted teeth (r = 0.68, p<0.0001; Spearman correlation analysis).

Sensitivity for detection of MS acquisition from swabbing different oral sites

Considering the four swabbed oral sites, the objective was to verify which of them was better for detecting MS acquisition. As shown in Figure 3, among the 4 sampled sites, dental plaque was an important site for detecting initial MS acquisition by infants, but without difference between saliva (p = 0.4077; Exact Fisher's test), and tongue dorsum (p = 0.1755; Exact Fisher's test). In relation to the mucous ridge, swabbing dental plaque showed a better sensitivity for detecting MS acquisition (p = 0.0330; Exact Fisher's test). Eight children showed the first positive culture in all of the sampled sites.

MS colonization characteristic at the end of the 24-month evaluation

Of the 33 infants selected for this study, 26 completed the 24-month evaluation, and were used for the MS colonization stability description. None of the 26 children remained MS-free for this period. Six children were classified as transiently colonized (4 children with only one MS positive culture and 2 children with non-consecutive MS positive cultures). Twenty children were classified as colonized (Table 2). Exact Fisher's test shows no difference between gender and the kind of colonization (p=0.2245) (Table 2).

During the 24-month-follow up, 1667 strains were identified as MS, 1340 isolated from the MSB samples

Table 2. The characteristics of MS colonization of child.

MS Colonization	Male	Female	Salivary level of mothers' MS (mean ± SD CFU/mL saliva)[δ]	Time of MS detection (mean ± SD) (months of children's lives)
PERSISTENT[α]	9	11	$2.71 \times 10^6 \pm 4.58 \times 10^6$	17.5 ± 4.8
TRANSIENT[β]	1	5	$2.45 \times 10^7 \pm 5.66 \times 10^7$	13.8 ± 4.3
NON-DETECTABLE[χ]	0	0	-	-

α Consistent isolation of MS after the first positive culture.
β Irregular isolation of MS after the first positive culture.
χ Repeated negative samples for MS.
δ During the first positive MS culture.

of children (1238 *S. mutans* – Sm and 102 *S. sobrinus* – Ss), and 334 from the MSB samples of mothers (306 Sm and 28 Ss).

Based on the results of PCR identification, at the end of the follow-up time 16 children were colonized with both *S. mutans* and *S. sobrinus*, 8 were colonized by *S. mutans* alone, and 2 were colonized by *S. sobrinus* alone.

Clinical findings

In relation to the clinical findings during the 24 months follow up, of the 26 studied children, 6 showed initial dental caries during the experimental period. The number of affected teeth ranges from 1 to 4. The means d-t score was 0.46 ± 1.02. The 6 children with dental caries were classified as permanently colonized. Four of these children were colonized with both *S. mutans* and *S. sobrinus*, and the other 2 were colonized by *S. mutans* alone.

Maternal factors

Among the 33 mothers, all had detectable MS levels ($6.24 \times 10^6 \pm 2.43 \times 10^7$ CFU/mL) during the initial phase of the study. Table 2 also resumes the characteristics of MS presence in mothers of permanently and transiently colonized infants.

The amount of MS in the mothers' saliva did not correlate significantly with their DMF-T ($r = -0.01$; $p = 0.95$; Spearman correlation analysis), or with the age of initial MS acquisition by their children ($r = 0.11$; $p = 0.56$; Spearman correlation analysis), or with the age of permanent colonization ($r = 0.22$; $p = 0.33$; Spearman correlation analysis).

DISCUSSION

A longitudinal study design was used to determine whether MS really has been established or whether it has only been a transient finding. While some investigations have mentioned its predentate presence[2,5,14-16] the majority of studies proposed that MS is not found until teeth erupt and create new potential niches and attachment sites for permanent oral bacterial colonization.[8-12] This report supports the concept that it was possible to identify the presence of MS in edentulous children, but in these cases, the microorganisms are only transient colonizers, because the presence could not be detected during the subsequent confirmatory sample collection. It is probably that the edentulous colonization determined in previous papers, involving only one sample period could also have been an incidental finding.[2,14,16]

On the other hand, the presence of MS in edentulous mouths, even during the confirmatory visit sample 3 months later, was related to special child characteristics like the presence of oral development nodules,[5] or breast feeding given on-demand.[15] Besides, some characteristics of the mother increase the risk for predentate colonization, such as women with periodontal pocketing, heavily visible plaque, subgingival calculus, high frequency of sugar consumption, and low socioeconomic level.[15] In this study, none of the maternal characteristics evaluated showed any relation to the time of MS acquisition, probably because all the mothers were heavily infected with MS, and presented similar caries prevalence. Other characteristics of the mothers should be evaluated.

With regard to the transient presence of MS in edentulous babies in this study, it is important to highlight that variations in microbiological methods with different species detection limits may explain discrepancies in the findings, in addition,[6,7] the finding that MS can colonize predentate children holds significance with regard to the etiology of early childhood caries, and should be further investigated.

With regard to our microbiological culture procedure, the technique of swabbing the different sites used, in addition to soaking the swab with saliva, yielded the maximum probability of positive MS culture. The choice of this practical procedure was based on studies involving the swabbing of saliva, tongue, oral mucosa and teeth of infants and adults.[5,8,15,31]

In agreement with Milgrom *et al.*[14] and Tanner *et al.*,[3] the detection of MS in the dorsum of the tongue (Figure 3) supports the idea that the tongue serves as a reservoir for tooth-associated species, but in this study, the collection of samples from saliva, tongue dorsum, and dental plaque did not show any difference related to sensitivity of acquisition detection. However, by reason of variability in detecting the first positive culture among the different sample sites (Fig. 3),

sample collection from more than one site can provide a more reliable detection of MS acquisition, which is in agreement with data from Grönroos & Alaluusua.

Several studies have suggested that the development of oral microbiota is related to the age of the child and number of erupted teeth. The number of erupted teeth shows a positive correlation with the time of initial MS acquisition, which is in agreement with previous studies.[8,10,17,18] The time of MS acquisition was also related to the time of tooth eruption, complementing the findings of some studies.[8,17,31]

The Caufield group data indicate that MS is transmitted over a finite period of time; the "window of infectivity". For 75% of the infants in their study, who became infected with MS, this window was between 19 and 31 months of age (median 26 months) and correlates with the emergence of primary molars.[31] Some studies suggested that MS might colonize children before the "window of infectivity" opens.[3,6,17] The findings in this study reinforce this information. In the studied sample, characterized by a low economic level, it was possible to verify that 88% (29/33) of the children showed at least one positive MS culture (13.8 ± 4.3 months of age), and 77% (20/26) were classified as permanently colonized by MS at the end of 24 months. The MS acquisition, considering only permanent colonization, was at between 14.4 and 20.3 months old (17.5 ± 4.8), significantly prior to the critical moment established in literature for MS implantation.[8] It is pertinent to point out that the acquisition time was checked with culture methods, so the sensitivity of the identification methods cannot influence the time of colonization, since they were used only for microorganism identification.

The precocity of acquisition was also related in some longitudinal studies. Masuda *et al.*[33] found colonized children between 13 and 24 months-old; Tedjosasongko, Kozai[19] studying 39 children found that the mean age of initial MS acquisition was 24.2 ± 12.3 months; to Wan *et al.*[20] the mean age was 15.7 months in a sample of 111 children followed up for 24 months. Considering studies in which MS colonization in low socio-economic children was based upon one collected sample, the precocity verified was the same. Mohan *et al.*[17] found MS in approximately 20% of the children less than 14 months old, including 18% of the children aged 6-9 months. Evidence of MS colonization was seen at as early as 10 months of age in 14% of the studied infants, 25% of those 12 months old and younger, and 60% of the 15-month old age group were colonized in the study of Karn, O'Sullivan & Tinanoff.[18] The proportion of children with MS in the study of Milgrom *et al.*[14] was 53.3% of the children aged 6-12 months, and 72.1% in the 13-24 months group. Tanner *et al.*[3] verified that 72% of the children as young as 18 months, and 75% of the children aged 19-36 months were colonized by MS.

Studies have shown that younger children with both *S. mutans* and *S. sobrinus* in the saliva have significantly more dental caries than those with either *S. mutans* or *S. sobrinus*[34,35] In some studies, *S. sobrinus* was not detected in association with caries, whereas in other studies, *S. sobrinus* detection indicated a higher risk of smooth-surface than did *S. mutans*. In the findings of this study, all the children, who developed dental caries, were permanently colonized, and four of them were colonized with both *S. mutans* and *S. sobrinus*.

Considering the importance of early MS infection as caries predictor, these findings show that early MS acquisition in this low-income population is possible, and suggest that the age of acquisition in general may need to be reconsidered. When examined separately, age of tooth eruption and number of erupted teeth were each found to be associated with time of MS acquisition.

In the next step of this research, the similarity between the MS strains, by genotypic approach, that were isolated in each of the pairs will be studied in order to demonstrate vertical and/or horizontal transmission. Additonal study will verify the stability or otherwise of the strains (genotypes) firstly acquired in children oral cavity during the follow-up study.

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