Periodontal Pathogen Colonization in Young Children by PCR Quantification – A Longitudinal Survey

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Introduction: *Periodontal diseases are among the leading causes of premature tooth loss in adults, but the microbiota associated with this problem is established over time in childhood.* ***Aim:*** *This longitudinal study aimed to verify the occurrence of periodontal pathogens in the oral cavity of children aged six, twelve, eighteen and twenty-four months through PCR quantification, correlating them with the oral microbiota of their mothers.* ***Study design***: *Saliva and oral biofilm samples were collected from mothers and children by using sterilized paper points. Furthermore, a questionnaire was applied in all periods to evaluate hygiene and dietary habits.* ***Results***: *A positive correlation was found between mother–child pairs in all periods. No correlation was observed between hygiene and dietary habits and occurrence of periodontal pathogens.* ***Conclusion***: *Early inclusion of children in preventive and biofilm control programs could contribute to preventing acquisition of aggressive pathogens.*

Key words: Saliva; Anaerobic Bacteria; Periodontal Diseases.

INTRODUCTION

The concept that children's dental care providers should work together with their parents is well established, as this promotes repeated consultations, and a positive relationship with the professionals, lasting into adulthood. Dentistry for babies became a trend in the contemporary dentistry, as its philosophy was based primarily on educational and preventive practices, even in the first months of life without any erupted teeth, determining risk factors for the different oral pathologies[1].

Periodontal diseases are among the leading causes of premature tooth loss in adults, but the microbiota associated with this problem is established over time in childhood. The most common manifestation in children and adolescents is gengivitis[2], and the inflammatory reaction that can generally be seen is dependent on microbial biofilm and bacterial aggression to the host. In most children, the process may begin at an early age and remains superficial. However, if there is an imbalance between aggression and the host response, involvement of the alveolar bone may also occur, resulting in the loss of connective tissue attachment[3].

As part of normal flora of oral cavity, there are a variety of microorganisms with different potential virulence factors[2]. In these groups, some gram-negatives and anaerobic species that play an important role in the etiology of periodontal disease[2] can be seen, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Eikenella corrodens* and *Treponema denticola*[4,5,6,7].

How and when these above-mentioned microorganisms colonize the oral cavity during childhood, are still unanswered questions in literature. Periodontal pathogens can also be transmitted by parents, especially mothers during childhood[8,9,10,11].

It is known that some children colonized by periodontal pathogens frequently show no signs of periodontal disease, and that there is a correlation between colonization and periodontal disease in mothers [6]. Dental clinicians must therefore be adept at diagnosis and management of periodontal disease in children and adolescents[12].

Considering all the factors that may be involved in the onset of gum disease in early childhood and the participation of mothers in this process, we understand that longitudinal studies related to the development of periodontal disease in children are rare in the literature; However, there are many studies with emphasis on children of school-going age. Therefore, the aim of this longitudinal study was to verify the occurrence of periodontal pathogens in the oral cavity of children aged six, twelve, eighteen and twenty-four months, by means of PCR quantification, correlating them with the oral microbiota of their mothers.

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MATERIALS AND METHOD

All procedures described below were previously submitted to the Research Ethics Committee of Araçatuba Dental School–UNESP for approval (Process FOA 2006 – 01470).

The sample of this research was selected during the first appointment , from among patients of the Babies Dental Clinic, who fulfilled the following criteria: edentulous children, enrolled in the program, who were not using antibiotics at the time of sample selection, patients without special needs and those whose mothers agreed to participate in the survey.

In all periods, a simple questionnaire was applied to mothers to evaluate the manner and frequency of oral hygiene habits. For each child, the mother was asked to write a diary of all food consumption in a day for a whole week. Food ingestion was classified as cariogenic or not, with association of nursing bottle. Subsequently, saliva and oral biofilm were collected for microbiological analysis and a simple periodontal exam was performed. No periodontal probe was used for this exam, as there are wide variations in gingival sulcus in children at this age caused by the tooth eruption process. In addition, the clinical inflammatory condition of tissues was evaluated.

In edentulous children, sampling was done in the morning, with sterile swabs used to gently collected 0.1 mL of gingival saliva. When children had erupted teeth, dental biofilm was collected with paper points that were placed in the gingival sulcus for 60 seconds. After this, samples were transferred to MilliQ water. For mothers, 0.1mL saliva was collected in tubes, and dental biofilm, with paper points. DNA was from clinical samples with the 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 performed with determined dilutions and by means of PCR equipment (Perkin Elmer, GeneAmp PCR System 2400), with determined cycles and temperatures. The amplification was performed in a volume of 25 ml, with 2.5 ml of 10 X PCR buffer, 1.25 ml of $MgCl_2$ (50 mM), 2.0 ml of dNTP (10 mM), 0.25 ml of *Taq* DNA polymerase (0.5 U), 1.0 ml of each starter (0.4 mM), 7 ml of sterilized Milli-Q ultrapure water and 10 ml of DNA (ng). Amplification was performed in PCR equipment (Perkin Elmer, GeneAmp PCR System 2400) programed as follows: 1 denaturation cycle at 94°C (5 min.); 30-36 cycles at 94°C (30s-1 min.), annealing temperature according to starter (Table 1), 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). Table 1 presents the specific starters for identification.

In the laboratory, clinical specimens were plated in VMGA III then submitted to serial dilutions in VMGA I and rates of 0.1 ml were plated as described below and the following incubation conditions [13]:

- agar Fastidious Anaerobe (FAA) enriched with yeast extract (0,5%), hemin (5 µg/mL), menadione (1 µg/mL) and 5% of defibrinated horse blood, incubated under anaerobic conditions (90 % N_2 + 10% CO_2), at 37° C, for 7 and 14 days, to isolate anaerobic specimens;
- agar TSBV, incubated under anaerobic conditions, at 37° C, for 2 and 4 days to isolate *A. actinomycetemcomitans*;
- Tryptic soy agar enriched with 5% of defibrinated horse blood, incubated under anaerobic conditions, at 37° C, for 2 and 3 days for aerobic and facultative anaerobic specimens;

The bacteria isolated were identified by means of their morphocellular, morphocolonial and biochemical-physiological characteristics.

Table 1 - Starters used in trials of bacterial DNA amplification.

Specific starters	Oligonucleotides	Temperature
Actinomyces sp.	5'-GGC KTG CGG TGG GTA CGG GC- 3' 5'-GC TTT AAG GGA TTC GCT CCR CCT CAC- 3'	53°C
A. actinomycetemcomitans	5'-GCT AAT ACC GCG TAG AGT CGG- 3' 5'-ATT TCA CAC CTC ACT TAA AGG T- 3'	60°C
C. rectus	5'-TTT CGG AGC GTA AAC TCC TTT TC- 3' 5'-TTT CTG CAA GCA GAC ACT CTT- 3'	60°C
E. corrodens	5'-CTA ATA CCG CAT ACG TCC TAA G- 3' 5'-ACT GTT AGC AAT CAA GTT GCC C- 3'	55°C
F. nucleatum	5'-ATT GTG GCT AAA AAT TAT AGT T- 3' 5'-ACC CTC ACT TTG AGG ATT ATA G- 3'	55°C
P. gingivalis	5'-AGG CAG CTT GCC ATA CTG CG- 3' 5'-ACT GTT AGC AAC TAC CGA TGT- 3'	40°C
P. intermedia	5'-TTT GTT GGG GAG TAA AGC GGG- 3' 5'-TCA ACA TCT CTG TAT CCT GCG T- 3'	57°C
T. forsythia	5'-GCG TAT GTA ACC TGC CCG CA- 3' 5'-TGC TTC AGT GTC AGT TAT ACC T- 3'	60°C
T. denticola	5'-TAA TAC CGA ATG TGC TCA TTT ACA T- 3' 5'-TCA AAG AAG CAT TCC CTC TTC TTC TTA- 3'	55°C

Statistical analysis

Hygiene models were evaluated by Fisher exact test. Distribution of microorganisms between children and their mothers was analyzed by Analysis of variance test. For oral conditions, the Chi-square test was applied; and interrelations between microorganisms and other variables were measured by Mann-Whitney test and Fisher exact test, and isolated for different periods by Spearman Correlation Test ($p<0,05$).

RESULTS

At six months, the authors observed that all microorganisms occurred more frequently in the mothers' saliva, except for *Fusobacterium periodonticum*–rarely detected–and *Treponema denticola*. The most frequently detected microorganisms were *Actinomyces*, *C. rectus* and *Fusobacterium nucleatum* (Table 2). Positive correlation was observed between mothers and children (over 80%) at six and twelve months. No influence of any other factors was seen (p= 0.317 to p= 1.0).

Table 2 - Occurrence of oral microorganisms related to periodontal disease in saliva of 6-month child and their mothers (N=50).

Microorganism	Occurrence N (%)			
	Children (culture)	Mothers (culture)	Children (PCR)	Mothers (PCR)
Actinomyces sp.	3 (6.0)	25 (50.0)	8 (16.0)	28 (56.0)
Aggregatibacter actinomycetemcomitans	1 (2.0)	9 (18.0)	2 (4.0)	7 (14.0)
Campylobacter rectus	8 (16.0)	15 (30.0)	13 (26.0)	15 (30.0)
Eikenella corrodens	4 (8.0)	18 (36.0)	4 (8.0)	20 (40.0)
Fusobacterium nucleatum	7 (14.0)	29 (58.0)	9 (18.0)	29 (58.0)
Porphyromonas gingivalis	2 (4.0)	8 (16.0)	4 (8.0)	8 (16.0)
Prevotella intermedia-nigrescens	4 (8.0)	20 (40.0)	6 (12.0)	22 (44.0)
T. forsythia	0 (0.0)	0 (0.0)	0 (0.0)	9 (18.0)
T. denticola	0 (0.0)	0 (0.0)	0 (0.0)	3 (6.0)

At twelve months, in the mothers a positive correlation of 83% was observed in the same microorganism when compared with the first period. But in the babies, detection of one specimen was coincident in 29% in culture, rising to 44% with PCR. *Eikenella corrodens* was the only bacteria that was statistically significant in this period (ANOVAmr, p=0,046). The Mothers' microbiota was more complex. Oral biofilm was stable (Table 3).

Table 3 - Occurrence of oral microorganisms related to periodontal disease in saliva and biofilm of 12-month children and their mothers. (N= 42).

Microorganism	Occurrence N (%)			
	Children (Saliva)	Mothers (Saliva)	Children (Biofilm)	Mothers (Biofilm)
Actinomyces sp.	13 (30.95)	27 (64.28)	14 (33.33)	27 (64.28)
A. actinomycetemcomitans	3 (7.14)	8 (19.04)	3 (7.14)	10 (23.80)
C. rectus	10 (23.80)	16 (38.09)	9 (21.42)	20 (47.61)
E. corrodens	8 (19.04)	20 (47.61)	7 (16.66)	18 (42.85)
F. nucleatum	10 (23.80)	27 (64.28)	10 (23.80)	24 (57.14)
P. gingivalis	3 (7.14)	8 (19.04)	2 (4.76)	8 (19.04)
P. intermedia	7 (16.66)	19 (45.23)	8 (19.04)	20 (47.61)
T. forsythia	0 (0.0)	7 (16.66)	0 (0.0)	8 (19.04)
T. denticola	0 (0.0)	3 (7.14)	0 (0.0)	6 (14.28)

At eighteen months there was a significant increase of *Actinomyces* sp. (ANOVAmr, p=0.015), *Eikenella corrodens* (ANOVAmr, p=0.005), *Fusobacterium nucleatum* (ANOVAmr, p=0.007) in saliva, compared with six months. Otherwise, in comparison with twelve months, the prevalence of *Actinomyces* sp. (ANOVAmr, p=0.012), and *Fusobacterium nucleatum* (ANOVAmr, p=0.029) increased. All specimens were detected more frequently in the mothers. Contamination was likely at 24 months. *Fusobacterium nucleatum*, had reached a stable colonization in 70% of the children. All microorganisms were more prevalent in the mothers (P<0.001) (Table 4).

Table 4- Occurrence of oral microorganisms related to periodontal disease in saliva and biofilm of 18-month children and their mothers. (N= 37).

Microorganism	Occurrence N (%)			
	Children (saliva)	Mothers (saliva)	Children (PCR)	Mothers (PCR)
Actinomyces sp.	13 (35.13)	20 (60.60)	12 (32.43)	22 (66.66)
A. actinomycetemcomitans	2 (5.40)	6 (18.18)	2 (5.40)	8 (24.24)
C. rectus	9 (24.32)	14 (42.42)	11 (29.72)	14 (42.42)
E. corrodens	9 (24.32)	19 (57.57)	10 (27.02)	19 (57.57)
F. nucleatum	17 (45.94)	20 (60.60)	18 (48.64)	21 (63.63)
P. gingivalis	3 (8.10)	7 (21.21)	3 (8.10)	8 (24.24)
P. intermedia	7 (18.91)	17 (51.51)	7 (18.91)	19 (57.57)
T. forsythia	1 (2.7)	6 (18.18)	1 (2.70)	8 (24.24)
T. denticola	0 (0.0)	2 (6.06)	0 (0.0)	3 (9.09)

Table 5- Occurrence of oral microorganisms related to periodontal disease in saliva and biofilm of 24-month children and their mothers. (N= 37).

Microorganism	Occurrence N (%)			
	Children (saliva)	Mothers (saliva)	Children (PCR)	Mothers (PCR)
Actinomyces sp.	18 (48.64)	20 (66.66)	21 (56.75)	20 (66.66)
A. actinomycetemcomitans	1 (2.70)	5 (16.66)	3 (8.10)	7 (23.33)
C. rectus	11 (29.72)	13 (43.33)	13 (35.13)	14 (46.66)
E. corrodens	10 (27.02)	13 (43.33)	9 (24.32)	17 (56.66)
F. nucleatum	19 (51.35)	18 (60.0)	19 (51.35)	18 (60.0)
P. gingivalis	3 (8.10)	7 (23.33)	4 (10.81)	7 (23.33)
P. intermedia	6 (16.21)	14 (46.66)	7 (18.91)	15 (50.0)
T. forsythia	0 (0.0)	5 (16.66)	2 (5.40)	7 (23.33)
T. denticola	0 (0.0)	2 (6.66)	0 (0.0)	3 (10.0)

Data showed the occurrence of three different types of pathogens: one group of specimens that was rarely detected in the same child in different time intervals, such as *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans*; another group that was frequent in the children but sometimes coincident in the time intervals, which suggested the need for reinfection, such as *C. rectus* and *P. intermedia*; and a third group with stable colonization, such as *S. mutans, S. sobrinus*, and *F. nucleatum*. *T. denticola* was not detected in saliva and biofilm of children in all periods (Table 5).

DISCUSSION

For practicing pediatric dentists, it is important to know that the presence of periodontal pathogens in the oral cavity of young children is a complex condition, coincident with oral colonization of mothers, but it seems to be transitory. Some pathogens responsible for periodontal disease may be found in children without being correlated to models of diet and oral hygiene.

The oral microbiota was established early and appeared to reflect, at least for some of its members, the characteristics of diet, hygiene habits and the attitude of parents and caregivers about preventing oral diseases[1]. From this aspect, the establishment of each microorganism in the oral environment, and their future relationship with the host appear to obey a concept of "window of infectivity"[14]; namely, there is a period in which the host, usually an infant exposed to a vehicle of transmission contaminated with a given microbial species, would be more easily colonized by this microorganism. This concept can be applied to the major specimens of the oral cavity.

Among the main microorganisms detected in the clinical samples, stands out the genus *Actinomyces,* whose colonization increased with eruption, was outstanding. Data from this study could, with appropriate limitations, be regarded as complementary to some previous researches[15]. They have shown that establishment and maturation of oral biofilm, particularly *Actinomyces* and members of red complex was slow and gradual. Tooth eruption created conditions for the development of acquired pellicle and biofilm formation–the main source of *Actinomyces* and other extracellular polymer levan carbohydrates[16], which could become sources of soluble fermentable carbohydrates to other members of the biofilm community. These rods detected in most samples of biofilm and each of the major species present could be observed in 40 to 50% of children at 6 months of age[17]. However, in this study, the occurrence of these microorganisms was significantly lower than those described in other studies[9,17]. At two months of age, 30% of the children had these microorganisms in their saliva, while at 24 months, over 90% of children harbored these gram-positive specimen. Similar values have been described[18] for 19-36 month-old children. These data differed significantly from those reported[19], which failed to detect these bacteria before tooth eruption.

A notable feature observed in this study was the stability of early colonization of oral cavity by *Actinomyces* in the present study. In this sense, a child colonized by these rods had more than a 70% chance of keeping the microorganism during the course of the evaluation time, and being shown to be carrier in the next collection of clinical specimens[18].

Occurrence of *A. actinomycetemcomitans* in the biofilm of children was quite modest, always below 9%, and no child showed presence of this organism in two subsequent collections, suggesting that the presence of this microaerophile would only be transitory in these children aged 24 months or younger, which was in agreement with some authors[20,21,22,6,3]. The mean age for occurrence of this pathogen was 3.5 years old[22].

Among the microorganisms evaluated here, *C. rectus* and *E. corrodens* were worth mentioning. By conventional PCR, the data presented in this study revealed the presence of *C. rectus* in the saliva of 26.0% of children aged 6 months and in 34% of their mothers, suggesting early colonization that has remained more or less constant over time, reaching 29.73% saliva samples at 24 months in children and 43.33% in mothers. In biofilm, the same phenomenon was present. In this sense, *Fusobacterium nucleatum* and Actinomyces, *C. rectus* were microorganisms with more stable colonization in the oral cavity of children and their mothers. Among the microorganisms studied, *C. rectus* was one that showed better correlation between the occurrence of dental biofilm and the presence of this microorganism in saliva[23, 24,25]. Few studies have reported the occurrence of this rod in the microbiota of both edentulous children and those in the stage of primary dentition. The data presented a slight rise in the prevalence of this microorganism in the biofilm of children, being 21.43% at 12 months to 35.14% at 24 months of age; below the values presented in another study[20], where 43% of the biofilm samples in children aged 6-18 months were positive, and this figure rose to 50% in the age group from 19-36 months. The prevalence of this ubiquitous microorganisms in the oral cavity increased in children over two years old, in whom these rods were harbored in 60% to 80% of individuals[21]. These data possibly reflect ethnical, cultural, social and geographic conditions that led to different patterns in other countries[20, 26, 27].

The discrepancies of this study appear to reflect possible periodontal conditions of the mothers and families, and the fact that children in this study were enrolled in an educational-preventive program that encouraged the restriction of sucrose consumption for the prevention of dental caries. Moreover, restriction of this carbohydrate is known to ultimately reduce the volume of biofilm[28], which is capable of affecting the distribution of this pathogen, since it is much more frequent in patients with poor biofilm control [29].

F. nucleatum was frequent in mothers as shown in the literature[25,15,27]. The data of this study, obtained by both culture and by PCR, confirmed the view that *F. nucleatum*, transmitted to edentulous children at an early stage, constituted one of the first anaerobic parts of human oral microbiota. In the early 1990s, 25% of children acquired this microorganism and the frequency of colonization had rapidly increased by the time they were 3 years old [29]. According to the results presented here, children between the ages of 12 and 18 months of life showed the highest values of colonization by this organism, and these remained more constant until the end of the study. In addition to being transmitted early, the results suggested stable anaerobic colonization in children, since the same children who harbored these microorganisms at 6 months continued to do so at 12, 18 and 24 months, among other children who were also colonized over this period of time[30,31].

Despite the importance of these microorganisms, a set of anaerobic microorganisms collectively referred to as the "Socransky red complex "[32] came to dominate research on the etiology and prevention of periodontal diseases[33,32]. The data suggested that even *P. gingivalis* and *T. forsythia* were rare and transitory in the saliva and biofilm of children until 24 months of age, which has also been demonstrated in scientific researches[33,20,26,35,21]. However, there are known factors that would not allow this microorganism to be established at an earlier stage in children with primary dentition. The same condition was observed with *T. forsythia*. In this study, at 18 months of age, only one child was positive for this rod, and

the same occurred with eight mothers. At 24 months, two infants were positive for this pathogen, but none of them presented it previously, suggesting that its presence is transient[35]. The presence of this bacteria in 14% of children aged between 6 and 18 months remained at this level between 19 and 36 months[18]. However, one study did not detect this anaerobic microorganism in children between 6 and 13 years of age[35]. Whereas, data from another study showed that it was retained in 5% of children aged between zero and four months old, and approximately 10% of children aged between two and five years, corroborating the conclusion that this bacteria was not part of the microbiota of children under the age of 24 months[21]. Few studies are in disagreement with this position[36], in which 65% of children aged from 5-9 years were carriers of *T. forsythia*. In our study, data showing the low prevalence of this microorganism in mothers and their children were in contrast with some results[35,18]. Moreover, other studies have questioned the role of this spirochete in the development of periodontitis, particularly the aggressive type [37].

The prevalence of *P. gingivalis* and *A. actinomycetemcomitans* seems to be more stable in older children, and values tend to be higher in children from 6-9 and 12-18 years old[38].

The literature has suggested that *P. intermedia*, a gram-negative anaerobic microorganism associated with periodontitis and gingivitis in humans, often found in children, would be transitory in the oral cavity[21,9,35]. However, in a more reliable manner than cross-sectional studies could offer, the results of the present study showed that approximately 20% of children and 40 to 50% of mothers were colonized by this bacteria.

With respect to the occurrence in children, our data are similar to those found in some studies[18,9,21], but in others, this microorganism was not detected. Comparison of these data suggested that peculiarities of populations of children, or more precisely, of the baby-parent pairs, may interfere with the stability of colonization.

Throughout the experiment, there was some occurrence of periodontal associations, so that the most positive samples were concentrated in some children. However, this more complex microbiota was transient and was not detected again in the next visit to Baby-Clinic. A similar phenomenon was described [37], in which there seemed to be an inter-relationship between the occurrence of the leading oral microorganisms, particularly those of the "Socransky red complex ".

Unquestionably there is need for early intervention to prevent tooth decay, since dietary and oral hygiene habits are formed early in the first years of the children's lives[38], because it is in this period that some of the most important microorganisms for the formation of biofilm are established. Since biofilm control is a measure that has already been adopted in dental care clinics for infants, measures to control biofilm–particularly the mothers'–are extremely important.

CONCLUSION

Irrespective of the microbiota detected, the periodontal tissues of children were in normal conditions;

Acquisition of periodontal pathogens occurred slowly; *Fusobacterium nucleatum* and A*ctinomices* showed premature establishment; otherwise *A. actinomycetemcomitans*, *T. forsythia*, *T. denticola* and *P. gingivalis* showed transitory occurrence;

P. intermedia, *E. corrodens* and *C. rectus* were prevalent, but colonization was not stable, suggesting transience.

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