Clinical status and detection of periodontopathogens and *Streptococcus mutans* in children with high levels of supragingival biofilm

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Abstract: Knowledge about the presence of some important oral pathogens is an important step in better identifying children at risk for periodontal and/or caries diseases in later life. The purpose of this study was to detect the presence of *Streptococcus mutans* (Sm), *Aggregatibacter actinomycetemcomitans* (Aa), *Campylobacter rectus* (Cr), *Porphyromonas gingivalis* (Pg), *Prevotella intermedia* (Pi), and *Tannerella forsythia* (Tf) in gingival biofilm samples from 196 children, and to assess whether any of these pathogens are more associated with gingival inflammation extension and the Decayed/Missing/Filled teeth (DMFT/dmft) index. The subjects presented plaque index greater than 80% and were divided in 3 groups according to the bleeding index (BI): I) Low bleeding (≤ 30%), II) Medium bleeding (31 – 59%) and III) High bleeding (≥ 60%). The presence of each pathogen was determined by PCR. The prevalence of Sm was 71.9% and the mean dmft/DMFT was 6.68. The prevalence in low, medium and high bleeding groups was 43.5%, 34.5% and 46.7% for Aa; 43.5%, 37.9%, and 36.7% for Cr; 99.1%, 100%, and 96.7% for Pg; 56.5%, 56.9%, and 66.7% for Pi; and 58.3%, 60.3%, and 56.7% for Tf, respectively. Pg (99.0%) was the most prevalent periodontal pathogen detected followed by Tf (58.7%), Pi (58.2%), Aa (41.3%) and Cr (40.8%). Our study indicated that in this high plaque index population studied, a high prevalence of Sm and high mean DMFT were observed. In addition, the presence of Pi was associated with the presence of inflammation ($P < 0.05$) whereas Cr was associated with periodontal health ($P < 0.05$).

Descriptors: Periodontal diseases; Dental caries; Dental plaque; Diagnosis.

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Introduction

Periodontal diseases are infections in which specific bacteria play an important role. The continuous dental plaque accumulation may result in an imbalance between pathogenic species and the host defense mechanisms, which may lead to gingival inflammation.[1] In fact, poor oral hygiene status has frequently been associated with a high prevalence or severity of periodontal disease.[2-4]

Current knowledge clearly shows that the microbiota associated with healthy periodontal sites is quite different from that of inflamed sites. Increased frequency of detection of *Aggregatibacter actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Tannerela forsythia* (Tf), *Treponema denticola* (Td), *Prevotella intermedia* (Pi), *Fusobacterium nucleatum* (Fn), *Campylobacter rectus* (Cr), and *Eikenella corrodens* (Ec) has been found in periodontal lesions.[5]

On the other hand, the bacterium *Streptococcus mutans* (Sm) is generally accepted to be the principal etiological agent of dental caries due to its high cariogenic potential.[6] Accordingly, higher levels of *S. mutans* have been associated with a higher risk for dental caries.[7] Thus, microbiological diagnosis of some of these specific pathogens should improve our ability to identify individuals or sites at risk for developing oral diseases.

Therefore, it is reasonable to believe that knowledge about the colonization process that takes place early in life is an important step in better identifying which children need more effective oral treatments in order to minimize the risk of periodontal and/or caries diseases in later life. In view of the controversial data available on the early infection by oral pathogens, the aim of the present study was to detect the presence of selected oral pathogens, using PCR, in gingival marginal biofilm samples from Brazilian children with high levels of supragingival biofilm, and to assess whether any of these pathogens are more associated with gingival inflammation extension and DMFT index.

Materials and Methods

Subject population

This study was conducted in accordance with guidelines of the 196/96 resolution of the National Health Council (NHC) and was approved by the University of Taubaté Ethics Committee (protocol # 0076/01). The children's parents or guardians received complete information regarding the objectives and procedures of the study and provided written informed consent.

One hundred and ninety six school children with mixed dentition (92 male and 104 female), ranging in age from 6 to 12 years (8.6 ± 1.4 years), from a public school in Taubaté (SP, Brazil) were enrolled in this cross-sectional study. The children in need of dental care were referred to the Dental Clinics of the University of Taubaté for appropriate treatment.

The children presented good general health and had not received treatment with antibiotics within the past 6 months prior to the study. In addition, the children selected for this experiment should present a high plaque index (minimum value of 80% for the Plaque Assessment Scoring System – PASS[8]).

Patients presenting systemic diseases, immunodeficiency and/or use of orthodontic or prosthetic devices were excluded from this study.

Clinical examination and microbial sampling

The clinical exam and microbial samples collection were performed in the children by two calibrated examiners. Intra- and inter-examiner agreement was high (minimum kappa value was 0.83).

Clinical examination comprised the determination of dental biofilm by PASS.[8] A total of 208 children were initially examined in this study. According to the cut-off point, plaque index > 80%, 12 children were excluded; therefore, 196 children (92 male and 104 female) completed the study. The bleeding index (BI)[9] was also evaluated. Dental status was assessed by the number of decayed, missing, filled teeth (dmft/DMFT), based on World Health Organization criteria (WHO).[10] For BI and dmft/DMFT, both dental arches were examined in their entirety. In accordance with BI, the children were divided into 3 groups: I) Low bleeding (≤ 30%), II) Medium bleeding (31% – 59%) and III) High bleeding (≥ 60%).

A pooled gingival marginal biofilm sample was

obtained from the mesial aspect of the same 5 PASS index teeth, i.e, first molars and right upper incisor. In absence of first molars or right upper incisor, second molars or left superior incisor were sampled. Before the collection procedure, cotton rolls were applied to prevent contamination of the sampling area with other oral fluids. The supragingival biofilm was gently removed with sterile cotton pellets and gingival marginal biofilm samples were collected using sterile paper points (Tanari #30, Tanariman Industrial Ltda., Manacapuru, AM, Brazil) inserted to the depth of the gingival sulci during 60 seconds. This procedure was done for each of the five sites previously selected and the paper points of each subject were placed in a same microtube with 1 ml of reduced Ringer's solution (0.9 g sodium chloride, 0.042 g potassium chloride, 0.025 g calcium chloride, 100 ml distilled water) on ice. The samples were stored at –80°C until the analyses.

Microbial analysis by Polymerase Chain Reaction - PCR

The bacterial cells in the microtube were dispersed using a Vortex™ and centrifuged for 3 min at 12,000 rpm. The genomic DNA was extracted from the bacterial pellet using a DNA purification Kit (InstaGene™ purification matrix, BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

The polymerase chain reaction (PCR) method was performed according to the protocol described by Cortelli *et al.*[11] (2008), using specific primers for *Streptococcus mutans*[12], *Aggregatibacter actinomycetemcomitans*,[13] *Porphyromonas gingivalis*,[13] *Prevotella intermedia*,[13] *Campylobacter rectus*[13] and *Tannerella forsythia*.[13]

Statistical analysis

Analysis of variance (ANOVA) was used for comparisons of the number of children in the different groups (Low, Medium and High bleeding). Student's *t post hoc* test was applied when appropriate. The prevalence of periodontopathogens among the groups was analyzed by the Chi-squared test (X^2). The Kruskal-Wallis test was used to compare *S. mutans* and DMFT index.

Finally, Pearson's test was used for the assessment of possible associations between *S. mutans* and the other periodontal pathogens (*Aa*, *Cr*, *Pg*, *Pi* and *Tf*). Statistical significance was established at 5%.

Results

Based on the Bleeding index (BI), the children were divided into three groups (Table 1). And, among children with high levels of supragingival biofilm we found a higher number of children with low bleeding scores ($p < 0.05$).

The microbial analysis by PCR indicated that 1 child (0.5%) was negative for all periodontal bacteria studied; 41 children (20.9%) were positive for one species, 42 children (21.2%) were positive for two species, 36 children (18.7%) were positive for three species, 29 children (14.8%) were positive for four species and 47 children (24.0%) were positive for all bacteria studied. The most prevalent periodontopathogen was *P. gingivalis* (99.0%), followed by *T. forsythia* (58.7%), *P. intermedia* (58.2%), *A. actinomycetemcomitans* (41.3%) and *C. rectus* (40.8%). Finally, the overall prevalence of *S. mutans* was 71.9%.

The analysis of the prevalence of each bacterium among the different groups indicated that no statistically significant difference ($p > 0.05$) was observed in the detection frequencies of *S. mutans* (71.3%, 77.6% and 63.3% for Low, Medium and High bleeding, respectively), *P. gingivalis* (99.1%, 100% and 96.7% for Low, Medium and High bleeding, respectively) and *T. forsythia* (58.3%, 60.3% and 56.7%

Table 1 - Demographic characteristics of the study volunteers (n = 196).

	Low Bleeding (≤ 30%)	Medium Bleeding (31 – 59%)	High Bleeding (≥ 60%)
N	108 children*	58 children	30 children
Gender	56 male / 52 female	24 male / 34 female	12 male / 18 female
Age	8.7 ± 1.4 years	8.6 ± 1.2 years	8.5 ± 1.5 years

* $p < 0.05$ by ANOVA, Student's *t post hoc* test.

for Low, Medium and High bleeding, respectively).

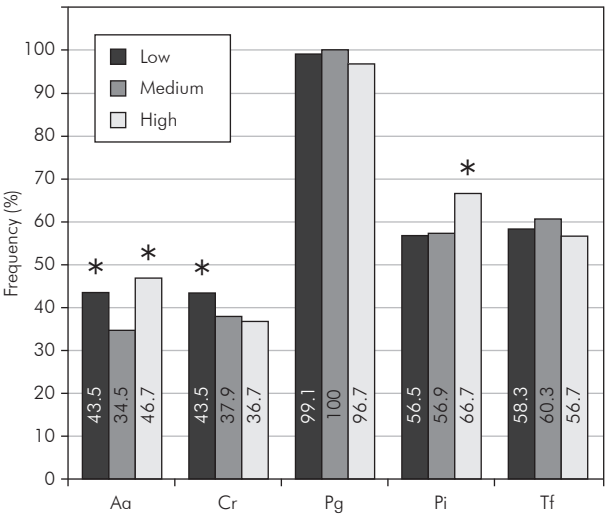
We also showed that a higher prevalence of *P. intermedia* was associated with gingival inflammation extension, since its detection frequency in the High Bleeding group (66.7%) was higher than in the Low (56.5%) and Medium (57.6%) bleeding groups. *C. rectus* was associated with periodontal health, because its detection frequency in the Low bleeding group (43.5%) was higher than in the Medium (37.9%) and High bleeding groups (36.7%). No statistical difference was observed between Low and Medium bleeding ($p > 0.05$).

The detection frequencies for all bacteria studied are shown in Graph 1. *A. actinomycetemcomitans* was more prevalent in the Low (43.5%) and High bleeding (46.7%) groups when compared to the Medium bleeding group (34.5%).

The bacterial frequencies in the low, medium and high bleeding groups were also evaluated according to the gender and age of the participants. There were no significant differences ($p < 0.05$) regarding the bacterial frequencies among these groups.

In order to determine the association between *S. mutans* and the periodontal bacteria studied (*A. actinomycetemcomitans*, *C. rectus*, *P. gingivalis*, *P. intermedia* and *T. forsythia*), Pearson's correlation coefficient (r) was calculated, and the results are summarized in Table 2. *S. mutans* did not show significant association with any periodontal bacteria. Among the periodontal pathogens evaluated, *P. gingivalis* showed the greater correlation coefficient with the presence of *S. mutans*, although with an extremely reduced value (r = 0.1626), which makes this finding irrelevant.

A high percentage of children showed positive samples for *S. mutans* (71.9%) and the population presented a mean dmft/DMFT value of 6.68. When the dmft/DMTF index was determined for each studied group (6.96, 6.30 and 6.78, respectively for low, medium and high bleeding), no significant difference ($p < 0.05$) was found. In addition, when the mean frequency of *S. mutans* was determined for each studied group (0.71, 0.77 and 0.63, re-



Graph 1 - Detection frequencies (%) of each bacterium (Aa – *A. actinomycetemcomitans*, Cr – *C. rectus*, Pg – *P. gingivalis*, Pi – *P. intermedia* and Tf – *T. forsythia*) observed in the different groups (Low, Medium and High bleeding). Asterisks indicate statistically significant differences between the groups; *$p < 0.05$ by the Chi-squared test (X^2).

Table 2 - Pearson's correlation coefficient (r) and p value for *S. mutans* and the other oral bacteria analyzed (Aa – A. *actinomycetemcomitans*, Cr – *C. rectus*, Pg – *P. gingivalis*, Pi – *P. intermedia* and Tf – *T. forsythia*).

	Aa	Cr	Pg	Pi	Tf
S. mutans	r = 0.0168	r = 0.0104	r = 0.1626	r = 0.0918	r = – 0.0629
	p = 0.8151	p = 0.8854	p = 0.0234	p = 0.2013	p = 0.3814

Table 3 - DMFT and medium frequency of *S. mutans* according to the studied group: Low, Medium and High bleeding.

	Low Bleeding (Mean ± sd)	Medium Bleeding (Mean ± sd)	High Bleeding (Mean ± sd)
DMFT	6.96 ± 3.78	6.30 ± 3.89	6.78 ± 4.27
S. mutans	0.71 ± 0.44	0.77 ± 0.41	0.63 ± 0.39

sd: Standard deviation.

spectively for low, medium and high bleeding), no significant difference ($p < 0.05$) was found either. Therefore, no statistical significant association was observed between the presence of *S. mutans* and the dmft/DMTF index of each studied group (Table 3).

Discussion

Periodontal diseases are due to the association between a plaque biofilm and the host responses. A specific group of bacteria, predominantly composed by Gram-negative, anaerobic microorganisms, is implicated in the initiation and progression of periodontal inflammation. The bacterial species *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *T. forsythia* have been identified as good predictors of future clinical attachment loss in susceptible hosts.[14]

In the present study, we showed that marginal biofilm samples from children between 6 and 12 years of age exhibited a high prevalence of oral pathogens. Interestingly, despite the high levels of supragingival biofilm and the high prevalence of periodontopathogens, the majority of the children analyzed (55.1%) presented a low bleeding score (< 30%) (Table 1). On the other hand, the high prevalence of *S. mutans* was compatible with the mean dmft/DMTF score (6.68). Considering the ages of the recruited children, between 6 and 12 years, the found DMFT/dmft value (6.68) can be considered high according to the mean DMFT, lower than 1, proposed by the World Health Organization for the age of 12 in the year 2010.[10]

P. gingivalis was the pathogen most frequently found (99%), followed by *T. forsythia* (58.7%), and *P. intermedia* (58.2%). *A. actinomycetemcomitans* (41.3%) and *C. rectus* (40.8%) showed a moderate prevalence. Similar observations were made by McClellan *et al.*[15] (1996) who used the same PCR analysis used in the present study, showing that *P. gingivalis* was detected in 40 to 50% of children ranging from 0 to 2 years old, and in 60% of teenagers aged 13 to 14 years.

No association between gingival inflammation and the presence of *T. forsythia* and *P. gingivalis* was found in the present study. Similar results were demonstrated by Kisby *et al.*[16] (1989) who showed that the prevalences of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* were approximately the same in healthy and gingivitis children between 8 and 10 years of age.

In accordance with the study of Gafan *et al.*[17] (2004), our study also failed to demonstrate any significant difference between healthy and gingivitis children between 5 and 9 years old with respect to the presence of *P. gingivalis* or *A. actinomycetemcomitans*. Surprisingly, these authors showed that *T. forsythia* was observed more frequently (2.3 times greater) in children without gingivitis compared to individuals with gingivitis. In our study, the presence of *A. actinomycetemcomitans* was not directly associated with health or disease status. Interestingly, the prevalence of *A. actinomycetemcomitans* was higher in the Low (43.5%) and High (46.7%) bleeding groups than in the Medium bleeding group (34.5%). Considering all the discrepancies found among the studies in the literature, further future investigations are still required in order to better understand the role played by each one of these cited species in periodontal disease in children.

In addition, our study evaluated the association between the presence of *S. mutans* and the identification of periodontopathogens in the marginal biofilm of children. Interestingly, *S. mutans* did not show significant association with any of the periodontal pathogens analyzed. Previous research has suggested that patients with periodontitis, particularly localized aggressive periodontitis, have minimal tooth decay.[18,19] Accordingly, a recent study by Fine *et al.*[20] (2007) has showed that *A. actinomycetemcomitans*-positive subjects, who predominantly had localized aggressive periodontitis (LAP), have a salivary factor that significantly reduces the survival of *S. mutans*. This finding suggested an explanation for the fact that the LAP group typically has minimal proximal tooth decay. The lack of any kind of association between the prevalence of *S. mutans* and the periodontopathogens in our study may be due to the high prevalence of plaque (plaque index greater than 80%) manifested by the population of children studied.

Our study is in accordance with a previous study by Tanner *et al.*[21] (1989) who demonstrated that putative periodontal pathogens colonization establish-

es in early years of life, therefore pointing out the need for more effective preventive dental programs.

Conclusion

It may be concluded that Brazilian children, ranging from 6 to 12 years of age, with high levels of supragingival biofilm, presented a high prevalence of periodontopathogens and *S. mutans*. It may also be concluded that in spite of the high levels of supragingival biofilm and high prevalence of periodontopathogens found in Brazilian children, ranging 6 to 12 years of age, the majority of them presented only mild inflammation revealed by a low bleeding score. On the other hand, high levels of *S. mutans* were compatible with poor dental status, i.e., high mean dmft/DMTF scores. Our study indicated that whereas *P. intermedia* was more associated with inflamed tissues, *C. rectus* was more associated with periodontal health.

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