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Prevalence of four putative periodontopathic bacteria in saliva of a group of Brazilian children with mixed dentition: 1-year longitudinal study

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Aim. This study aimed at evaluating the prevalence of putative periodontal pathogens (*Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella nigrescens*, *Treponema denticola*) in saliva of children with mixed dentition at two different time points, correlating these findings with a clinical parameter of gingival health.

Design. Polymerase chain reaction (PCR) detection was used to determine the prevalence of these bacteria in saliva of 64 children in 2003 and 60 children in 2004. Gingival health was assessed by gingival index.

Result. Sixty-two (96.9%) and 50 (83.3%) children presented low gingival inflammation, whereas 2 (3.1%) and 10 (16.7%) had moderate scores in 2003 and 2004, respectively. Majority of the children (81.3% in 2003 and 73.3% in 2004) had detectable levels of at least one of the bacteria. The prevalence found was of 4.7% and 1.7% for *A. actinomycetemcomitans*, 6.3% and 8.3% for *P. gingivalis*, 23.4% and 48.3% for *P. nigrescens* ($P < 0.05$), and 71.9% and 50% for *T. denticola* ($P < 0.05$) in 2003 and 2004, respectively. No significant relationship between gingival index and presence of these bacteria and combination of different species was found.

Conclusion. A high percentage of children harboured at least one of the putative periodontal pathogens in saliva, but presented periodontally healthy conditions.

Introduction

Periodontal disease comprises a group of conditions that affect gingiva, periodontal ligament, cementum, alveolar bone, and tissue structures that support the teeth[1]. The predominant form of periodontal disease in children and adolescents is gingivitis, which is a nonspecific inflammatory reaction of the marginal gingiva with no accompanying destruction of periodontal tissue[1,2]. Some of the major periodontopathic species are *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*[3–6]. Other species, however, have also been related to the disease, such as *Prevotella nigrescens* and *Treponema denticola*, among others[5–8].

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It is of particular importance to investigate the early colonization of periodontal pathogens, which can be an important approach for prevention and treatment of periodontal disease. Permanent colonization (or frequent acquisition) is difficult to distinguish from transient detection of microorganisms. Especially in children with mixed dentition, it is possible that these pathogens remain in the area surrounding exfoliating primary teeth and continue to survive in the gingival sulcus around permanent teeth in the mixed dentition stage[9–11]. There are few studies, however, that examine microbial colonization and gingival health during childhood in healthy populations[12]. Thus, the relationship between clinical parameters such as gingival index (GI) and the distribution of several periodontal pathogens in children is not yet well understood[13].

In the study of the oral bacterial community, saliva is a suitable sample as it is considered that it contains a variety of bacteria from different

oral sites (mucosa, tongue, supra- and subgingival plaque, gingival sulcus, etc.)[14–16]. The use of saliva for periodontal diagnosis has been the subject of considerable research activity, and proposed markers for disease include proteins of host origin, phenotypic markers, host cell, hormones, bacteria and bacterial products, volatile compounds, and ions[5]. It has been suggested that microorganisms in dental plaque can survive in saliva, and can utilize salivary components as a substrate. Moreover, the salivary levels of the periodontal pathogens may reflect the periodontal status of the patient[5]. Thus, whole saliva sample may offer a rapid, noninvasive and easy source for bacterial examination by employing a sensitive detection technique such as polymerase chain reaction (PCR)[5,14,15].

Therefore, the purpose of this study was to determine whether the presence of *A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens*, and *T. denticola* in the saliva of Brazilian children with mixed dentition could be correlated to the severity of gingival inflammation determined by GI at two different time points (with an interval of 1 year).

Methods

Study population

The institutional Ethics Committee approved the protocol of this study (# 19/2003). Sixty-four children from a public elementary school from Bauru, São Paulo State, Brazil, were recruited as study subjects in 2003. The age and gender of study children were recorded. The selection criteria were that the children possessed good general health and the presence of both primary and permanent teeth in the oral cavity. None of the subjects had undergone treatment with antibiotics within the past 6 months before the study. The parents or guardians of the children received detailed information concerning the nature and the procedures involved in the study and signed informed consent forms.

After 1 year, all the subjects were reassessed. Four children were lost to follow-up. All the following procedures were performed at both the initial and follow-up study (November of 2003 and November of 2004).

Gingival index

A clinical exam was performed in the children at a dental office with adequate light conditions, using a dental mirror and wood spatula. Through the GI[17], the severity of gingival inflammation was evaluated and judged to be low (scores from 0.1 to 1.0), moderate (1.1–2.0), or high (2.1–3.0) in degree[17,18]. Examinations were performed by two previously calibrated operators (kappa value of 0.88 for interexaminer consistency).

Saliva collection

Expectorated whole unstimulated saliva from each child was collected[16,19]. The children were asked to expectorate saliva into a sterile beaker over a period of 5 min. Approximately 2.0 mL were transferred to a sterile microcentrifuge tube, and frozen at −20 °C for later analysis[19].

DNA extraction

Preliminary studies indicated that whole saliva inhibited PCR amplification[19] (confirmed in our laboratory). Therefore, 500 μL of saliva samples were diluted 1:2 with sterile water and collected by centrifugation at 10 000 ×***g*** for 5 min in a microcentrifuge at 4 °C. The supernatant was discarded and the resulting pellet was washed two more times with 1 mL of sterile water. The pellet was reconstituted with 100 μL of water and 100 μL of InstaGene Matrix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and incubated at 56 °C for 30 min. The samples were then vortexed and boiled for 10 min. After centrifugation to remove unbroken cells and large debris (10 000 ×***g*** for 3 min), the supernatant was used for PCR analysis.

PCR conditions

A total of 50 μL of PCR mixture contained 10 μL of DNA sample, 5 μL of 10 X PCR buffer, 1.25 unit of *Taq* DNA polymerase, 0.2 mM each of deoxyribonucleotides, 1.0 μM of each primer, and 1.0 mM of $MgCl_2$ for *A. actinomycetemcomitans* and *P. nigrescens* or 1.5 mM of $MgCl_2$ for *P. gingivalis* and *T. denticola*[20]. As positive controls,

isolated DNA from *A. actinomycetemcomitans* ATCC 29522, *P. nigrescens* ATCC 33563, *P. gingivalis* ATCC 33277, and *T. denticola* ATCC 33520 were tested with the species-specific primers. Ubiquitous primers were also used as a positive control for the PCR amplification. For each set of primers, PCR was performed on sterile water to check for DNA contamination (negative controls). Considering that *Prevotella intermedia* is a phylogenetically close species from *P. nigrescens*, *P. intermedia* ATCC 25611 was also used as a negative control. Specificities of all primers were previously investigated[7,20,21] and primer sequences were compared with similar sequences of the reference organisms by BLAST search (http://ncbi.nlm.nih.gov/blast/). To further test the specificity of the primers, total DNA from all bacterial strains cited above was used with each of the primer pairs. The sequences of the primers and the temperature profiles were previously described[19,20].

PCR amplification products (9 μL) were analysed by 2% agarose gel electrophoresis. The agarose gels were stained with 0.5 μg/mL ethidium bromide and photographed under ultraviolet light. A 100 bp DNA ladder served as the molecular weight marker.

The detection limit of the PCR method was determined using DNA from known numbers of target organisms (10–10^7 cells per PCR as determined by viable-cell counts).

Statistical analysis

A descriptive analysis was separately performed with the data from both assessments. The results were evaluated by Fisher's exact test in order to verify possible correlations between the condition of gingival inflammation (low, moderate, or severe) and the prevalence of each studied bacteria and the combination of species. Wilcoxon and McNemar tests were also used in order to determine significant differences between data from both assessments. Statistical significance was established at 5%.

Results

Demographic data and clinical measurements of GI in 2003 and 2004 are presented in Table 1. A statistically significant variation of gingival inflammation condition was observed, with 15% of 60 children turning from low to moderate scores ($P < 0.05$, McNemar test). The mean value ± standard deviation for GI was 0.856 ± 0.133 and 0.992 ± 0.094 in 2003 and 2004, respectively. A mild increase of GI was observed in most of the children, and this increase was statistically significant ($P < 0.05$, Wilcoxon test).

The primers for *A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens*, and *T. denticola* demonstrated specific amplification of each bacterial species and did not amplify DNA from other species, thus validating primers specificity (data not shown). In contrast, no PCR product was found when performing the experiments with the negative controls. Based on ethidium bromide-stained gels, the detection limit of PCR was estimated to be approximately 10^2–10^3 bacterial cells per PCR (data not shown).

PCR results indicated that 18.8% and 26.7% of the children presented none of the four periodontal pathogens studied in 2003 and 2004, respectively. No relationship between the gender of the children and bacteria detected in their oral cavities was found (data not shown).

Table 1. Demographic data and clinical measurements of gingival index in the first (2003) and second assessment (2004).

	2003	2004
Demographic data		
Number of subjects	64	60
Gender	34 girls 30 boys	31 girls 29 boys
Mean age ± standard deviation (range)	9.07 ± 0.35 (8.33–9.91) years	10.1 ± 0.35 (9.33–10.91) years
Gingival index		
Children with low scores	96.9%	83.3%
Children with moderate scores	3.1%	16.7%

Table 2. Relative (%) and absolute frequencies (number in parentheses) of *A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens*, and *T. denticola*, as well as each species combination, in saliva samples of 64 children in the first assessment (2003) and of 60 children in the second one (2004).

Periodontopathic bacteria	Frequency of detection	
	2003 *n* = 64	2004 *n* = 60
A. actinomycetemcomitans	4.7% (3)	1.7% (1)
P. gingivalis	6.3% (4)	8.3% (5)
P. nigrescens	23.4% (15)	48.3% (29)
T. denticola	71.9% (46)	50.0% (30)
A. actinomycetemcomitans and *P. gingivalis*	1.6% (1)	0.0% (0)
A. actinomycetemcomitans and *P. nigrescens*	4.7% (3)	0.0% (0)
A. actinomycetemcomitans and *T. denticola*	4.7% (3)	0.0% (0)
P. gingivalis and *P. nigrescens*	3.1% (2)	5.0% (3)
P. gingivalis and *T. denticola*	3.1% (2)	5.0% (3)
P. nigrescens and *T. denticola*	17.2% (11)	28.3% (17)
A. actinomycetemcomitans, *P. gingivalis*, and *P. nigrescens*	1.6% (1)	0.0% (0)
A. actinomycetemcomitans, *P. gingivalis*, and *T. denticola*	1.6% (1)	0.0% (0)
A. actinomycetemcomitans, *P. nigrescens*, and *T. denticola*	4.7% (3)	0.0% (0)
P. gingivalis, *P. nigrescens*, and *T. denticola*	3.1% (2)	3.3% (2)
A. actinomycetemcomitans, *P. gingivalis*, *P. nigrescens*, and *T. denticola*	1.6% (1)	0.0% (0)

The prevalence of each single species and the species combinations found in this population is shown in Table 2.

Regarding the four species of bacteria studied in the first assessment, 81.3% of the children harboured at least one species, 17.2% at least two, and 6.3% at least three. Additionally, 64.1% harboured exactly one species, 10.9% two species, 4.7% three, and 1.6% four species. In the second year, 73.3% of the children harboured at least one of the species, 31.7% at least two, and 3.3% at least three. Lastly, 41.7% harboured exactly one species, 28.3% two species, 3.3% three, and no child harboured all four species in the follow-up year.

No significant correlation between the condition of gingival inflammation and the prevalence of each of the four periodontal pathogens, as well as their combinations in either or both periods of assessment, was found ($P > 0.05$, Fisher exact test).

The concordance for the presence of *A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens*, and *T. denticola* at the first and second samplings is shown in Fig. 1. No significant association between colonization at the first and second

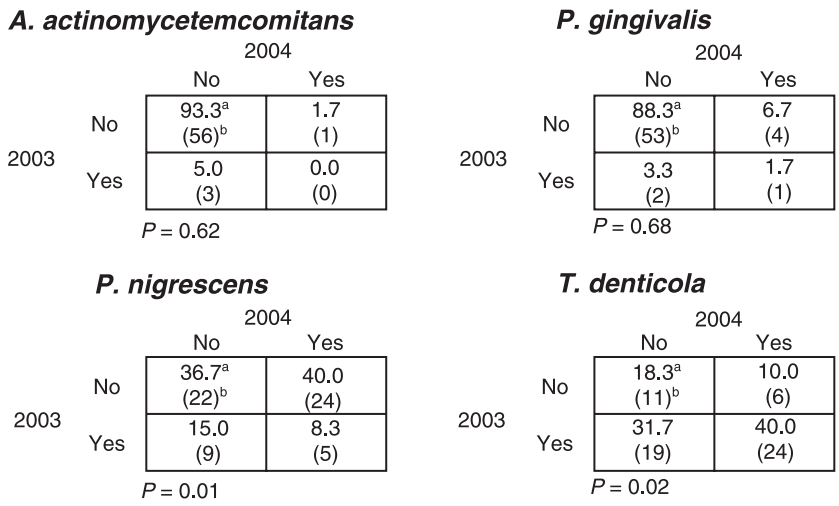

Fig. 1. Matrix of concordance of colonization between the first and second samplings for *A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens*, and *T. denticola*. (a) Percentage of children in relation to the total; (b) observed number of children (values in parentheses).

sampling times was seen for *A. actinomycetemcomitans* and *P. gingivalis*. A statistically significant increase in the number of children harbouring *P. nigrescens*, as well as a statistically significant decrease in the number of children harbouring *T. denticola* was seen.

Discussion

The analysis of the severity of gingival inflammation in our study demonstrated only mild or moderate manifestations of disease. This is in agreement with the results found in the published works for children of different ages, socioeconomic levels, and ethnic groups[18,22]. According to Jenkins and Papapanou[23] and Albandar and Tinoco[2], mild and moderate forms of gingival inflammation are almost a universal finding in young people. Oral hygiene level may also relate to the severity of gingival inflammation[24]. We did not employ oral hygiene level (oral hygiene index or plaque control record) because, unfortunately, the principal and teachers of the school did not allow the children to be out of the classroom for this additional procedure.

Comparing our results from 2003 with those from 2004, a statistically significant variation of gingival inflammation condition and an increase in GI scores in majority of the children were observed. Early evidence demonstrates that both the prevalence and severity of periodontitis increase with increasing age, suggesting that age may be a marker for periodontal tissue support loss[3]. In our study, however, this variation in a single clinical parameter in children is not sufficient to prove the existence of a direct relationship between the increase of age and the worsening of gingival condition, especially as the interval between both assessments was just one year. Therefore, future evaluations would be necessary in order to confirm this relationship.

One major aspect of periodontal disease prevention is the identification of subjects at high risk, and one of the major risk indicators is the presence of potential periodontal pathogens. Even with gingivitis being considered a common finding in children, its monitoring in childhood by the early detection of putative microorganisms related to periodontitis could have considerable impact on prevention and, likely, on modulation or elimination of the disease in adults. Obviously, there is no direct proof that paediatric colonization by these bacteria predisposes an individual to adult periodontitis; however, the earlier infection is established, the greater the opportunity the microorganisms have to establish themselves[12].

Our study reported the use of 16S rRNA-based PCR detection of four major putative periodontal pathogens. Although the relationship between the colonization of periodontopathic bacteria and sex hormones had been previously reported[6], this finding was not observed in our study. It is possible that secretion of sex hormones may influence the growth of some bacteria in the oral cavity of females[6]. Therefore, future investigations of this population, when the girls will probably be in the age of menstruation, might be helpful to assess the relationship between sex hormones and the detection frequency of *A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens*, and *T. denticola*.

The low prevalence of *P. gingivalis* observed is probably due to the fact that this bacterium apparently becomes more stable in the late teenage years[11], and its presence is most strongly associated with the progression of gingivitis and onset of periodontitis in adolescence (12 years and older)[12]. Microbiological culture studies indicate that *P. gingivalis* is not frequently found in children at an early age because the oral environment does not favour its colonization until after puberty[4,10,25,26]. Molecular biology techniques confirmed the occurrence of *P. gingivalis* in 5% of salivary samples of Finnish children aged 5–10 years old with a healthy periodontium[14]. Ashimoto *et al*. detected this bacterium by PCR in 14% of children around 7 years of age[20], Ooshima *et al*. reported a detection frequency of 10% in children aged 2–15 years old[21].

Other bacteria, such as *P. nigrescens*, have been isolated from the oral cavity of periodontally healthy young children in low amounts more frequently than *P. gingivalis*[23,27], as it is considered a frequent inhabitant of periodontal pockets[19]. From 2003 to 2004, we found a statistically significant increase in the detection rate of *P. nigrescens*. In a recent study[21], *P. nigrescens* was first detected in subjects at the age of

4 years, and its detection rate increased with age, with the detection frequency reaching 73% in 14-year-old children. Mättö *et al*. detected *P. nigrescens* in four of six children of 2–3 years old[27]. Similarly, Conrads *et al*. detected *P. nigrescens* from preschool children up to young adults with a normal periodontium[28]. They also reported that one could draw the conclusion that *P. nigrescens* is likely to be a member of the normal biofilm, colonizing the gingiva at a very early age. In contrast, Tamura *et al*. rarely found this species in children aged 2–12 years old (less than 5% of the children)[6].

Our findings showed a significant decrease in the detection of *T. denticola*. This is in agreement with the findings of Umeda *et al*., who detected *T. denticola* in almost 60% of children with mixed dentition[6]. Barron *et al*., using monoclonal antibodies, detected *T. denticola* in 50% and 70% of children 2–4 and 5–11 years old, respectively[29]. They also speculated that it may be the first treponema to colonize the deciduous dentition. The detection rate of this species, however, was lower in the work by Tamura *et al*. (16.8%)[16]. In addition, Okada *et al*. reported that this species is rarely found established in the oral flora of periodontally healthy gingiva, suggesting that this colonization may be transient[30].

Our findings for *A. actinomycetemcomitans* detection were similar to those obtained by other authors[11], who reported a prevalence of 4.8% of *A. actinomycetemcomitans* in healthy children aged 2–12 years, and 6.8% in those with gingivitis. Umeda *et al*. detected this bacterium in oral cavity of 1.8% of the children[6]. Although Morinushi *et al*.[12] have reported the close association between colonization by *A. actinomycetemcomitans* and the onset of gingivitis in early childhood (3–7 years) and its severity in adolescence, other authors agree that this species is rarely present in the oral cavity of children[11] or individuals with periodontal health[31]. These data are in disagreement with the finding of Tamura *et al*., who reported a detection rate of 42.5% for this species[16]. *A. actinomycetemcomitans* has often been identified in young persons showing severe attachment loss and/or rapid disease progression[32].

Although single species of bacteria have been identified as risk factors for periodontal tissue destruction, most infections in periodontal pockets appear to be mixed infections. Certain combinations of bacteria species have been implicated in the production of a pathogenic potential necessary to cause progression from gingivitis to destructive periodontitis[13,22]. In our study, no positive association among bacterial combinations was found, which is in accordance with the results from Kimura *et al*. who found no positive association in periodontally healthy children[7].

In this study, there was no statistical correlation between gingival inflammation condition (low or moderate) and the prevalence of each of the four bacteria or their combinations in saliva samples at either time point. These results suggest that although gingival inflammation slightly increased over the 1-year interval, the frequency of persons testing positive to the bacteria decreased. Additionally, half of the children ($n = 5$) with increased GI scores increased bacterial detection frequency, whereas the other half of these children ($n = 4$) decreased bacterial detection frequency. This finding can be explained by the fact that gingival inflammation is a nonspecific infection that can occur by any change in the composition of the bacterial species residing in the biofilm. Despite the lack of statistical correlation, however, between clinical parameters regarding periodontal health condition and the frequency of putative periodontal pathogens, a large number of children harboured at least one of the four species of bacteria studied. Therefore, it may be important to identify the determinants of oral colonization of these periodontopathic microorganisms, as they may persist in the oral cavity of untreated children for prolonged periods, thus increasing the risk of developing destructive periodontal disease in the future.

Although the great majority of children assessed in 2003 and 2004 presented at least one species of periodontal pathogens in saliva, the clinical parameter of periodontal health (GI) showed only low to moderate gingival inflammation in both periods. PCR detection methods and whole saliva samples provide a rapid and accurate method to screen for the presence of periodontal pathogens in oral cavity of children with mixed dentition.

What this paper adds
- The presence of periodontal pathogens in saliva of children with mixed dentition does not necessarily mean the existence of active periodontal disease.

Why this paper is important to paediatric dentists
- PCR detection methods and whole saliva samples provide a rapid and accurate method to screen for the presence of periodontal pathogens in oral cavity of children with mixed dentition.

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