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Detection of Periodontal Pathogens in Newborns and Children with Mixed Dentition

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Abstract

We report the age-related prevalence of red complex periodontal pathogens, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, along with 4 strains of orange complex pathogens. The bacteria present in samples isolated from tongue, cheek and subgingival sulcus in edentulous newborns and children with mixed dentition were monitored by PCR. *P.gingivalis* was not detected in any site of any subject in the two groups tested. However, *T.denticola* was not only found in the 6–13y group, but also in edentulous newborns at a relatively high prevalence, indicating non-dentition-related colonization by *T.denticola*. *C.rectus*, *P.intermedia*, *T.forsythia*, *E.corrodens* and *P.micra* was found in the oral cavity of most subjects belonging to the 6–13y group compared to newborns, suggesting a pronounced association between these colonizing bacteria and the presence of teeth. There was also a strong relation between the *T.denticola* and *T.forsythia* for their prevalence in the subgingival sulcus of the 6–13y group ($p<0.0001$), but not in the other sites tested, suggesting that colonization of dentition-related *T.forsythia* may associated with the increased prevalence of non-dentition-related *T.denticola* in the subgingival sulcus. Overall, these results suggest that dentition is a key determinant of bacterial colonization, especially orange complex bacteria and the red complex bacterium *T. forsythia*.

Keywords

Prevalence; periodontal disease; dentition; newborns; children; periodontal pathogens

Introduction

Periodontopathogenic bacteria are transmissible among family members, and children seem to acquire the periodontal pathogens predominantly from their parents [1]. Development of the habitation of indigenous microbiota begins on the surfaces of the human body after birth, when infants are exposed to continuous person-to-person and environmental contact with microbes [2]. Early childhood years are the critical period for the acquisition of certain bacteria, and close household contacts may be the source of acquisition of bacterial colonization for infants and children [3]. Our group has previously reported that initial colonization by five selected periodontal pathogens, including *C. rectus*, *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, and *T. forsythia*, could be detected in distinct age-related groups from newborns to elders [4]. *C. rectus* was detected in the oral cavity as early as 0–4 months of age, suggesting that oral colonization of this bacterium may not require niches provided by teeth [4]. In contrast, colonization by *P. gingivalis* appears to start when mixed dentition shifts completely to permanent dentition (>13 years of age), while colonization of *P. intermedia* and *T. forsythia* appeared to start at earlier ages, during the period when permanent teeth start erupting (6–12 y old) [4]. Although it remains unclear exactly how or when periodontal pathogens colonize healthy subjects, an understanding of age-related initial colonization of periodontal pathogens, especially *P. gingivalis* and *T. forsythia*, would provide a potentially useful diagnostic tool in the early detection and prevention of periodontitis in healthy individuals.

Based on their association with severe forms of periodontal disease, *P. gingivalis*, *T. forsythia*, and *T. denticola* are all categorized in the red complex bacterial taxa by Socransky et al [5]. *T. denticola*, a motile and highly proteolytic Gram-negative oral spirochete is thought to be a late colonizer during plaque biofilm formation [5]. Because of its motile nature, *T. denticola* appears to require adhesion to other oral bacteria, especially *P. gingivalis*, according to *in vitro* assays, in order to form biofilm [6]. The importance of this in the context of age-related initial colonization of periodontal pathogens is derived from the premise that some earlier oral colonizers may facilitate the biofilm formation of *T. denticola*. To date, however, both the time and sites of initial childhood *T. denticola* colonization, in association with other periodontal pathogens, remain unclear.

Bacteria of the orange complex, such as *C. rectus*, *P. intermedia*, *E. corrodens* and *P. micra*, are more tightly associated with red complex microorganisms than other color-coded (yellow, green or purple) complex bacteria because they are found in greater numbers in diseased sites and in more advanced forms of periodontal disease [5]. As such, it is conceivable that orange complex bacteria may be associated with the initial colonization of red complex bacteria in the oral cavity. Nonetheless, this secondary question involving the possible interaction between red and orange complex bacteria in their initial colonization in the oral cavity of children has not been addressed. Moreover, most recently published studies monitored the colonization of some of orange complex bacteria, but without analyzing the possible inter-bacterial interaction between red and orange complex bacteria [4, 7, 8].

Therefore, the aim of this cross-sectional study was to investigate the age-related prevalence of eight periodontal pathogens, including *Campylobacter rectus*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Tannerella forsythia*,

Eikenella corrodens, *Treponema denticola* and *Parvimonas micra*, in edentulous newborns and children with mixed dentition. The prevalence of three bacteria, *Treponema denticola* from the red complex and *Eikenella corrodens* and *Parvimonas micra* from orange complex, which were not included in our previous study [4], were investigated in the present study. Statistical analyses of data addressed not only the age-related prevalence of bacteria, but also the possible inter-bacterial association between the prevalence of, for example, orange complex bacterium and red complex bacterium, because it is hypothesized that orange complex bacteria cooperate the initial colonization of red complex bacteria in the oral cavity.

Methods

The present cross-sectional study included newborns up to four months of age recruited from the University Hospital of Taubaté (São Paulo, Brazil) and children from 6 to 13 years of age recruited from the Department of Dentistry at the University of Taubaté.

Data and personal information related to medical and dental histories of the subjects were obtained via responses to a questionnaire submitted by prospective subjects or their parents. The legal guardians of study subjects signed an Informed Consent form, which was approved prior to the start of this study by the Institutional Committee on Research Involving Human Subjects of the University of Taubaté (protocol 109/08).

The following subjects were excluded from the study: (1) subjects with uncontrolled systemic diseases; (2) subjects with any immunologically compromised condition; (3) newborns that had been given antibiotics; (4) subjects who were preterm births or had low birth weight. Specifically, among children 6–13 years of age, we also excluded (5) subjects who had taken antibiotics within the six months prior to the clinical and microbial examination, (6) subjects who use orthodontic devices; (7) subjects who underwent periodontal treatment 12 months before the beginning of the study; (8) subjects who needed antibiotics prophylaxis for dental treatment; and (9) subjects with no permanent first molars and central incisors.

For newborns, the oral examination was only carried out by visual inspection. For children, the clinical examination consisted of the Visible Plaque Index (VPI) and the Gingival Bleeding Index (GBI) according to Ainamo and Bay [9]. One trained and calibrated examiner conducted all clinical measurements and collected the microbial samples.

Microbial samples of the left side of the cheek mucosa and the dorsum of the tongue were obtained from all subjects. These samples were taken from areas of approximately 1 cm² using a swab with a reduced Ringer's solution (Oxoid Ltd., Basingstoke, Hampshire, UK), rotated six times. Each swab was transferred into a microtube containing reduced Ringer's solution (1 ml). In addition, a pooled subgingival sample was collected from each child aged 6 to 13 years from the mesio-buccal aspect of first molars (n=4 molars/subject) and mesial incisors (n=2 incisors/subject), using sterile paper points inserted into the depth of the sulcus after the removal of supragingival plaque using sterile curettes. Sixty seconds after placement in the sulcus, the paper points were immediately placed in a microtube containing reduced Ringer's solution.

The bacterial cells in the microtube were dispersed using a vortex mixer at maximum speed for one minute, and the resulting bacterial suspension was saved in a freezer at –80°C until laboratory processing. The presence of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, *C. rectus*, *E. corrodens*, *T. denticola* and *P. micra* was determined by polymerase chain reaction (PCR), as described below.

The bacterial suspensions were thawed and centrifuged at $12,000 \times \text{r.p.m.}$ for 3 min, and the DNA was extracted from the bacterial pellet using a DNA isolation kit, following the manufacturer's instructions (InstaGene purification matrix; BioRad Laboratories, Hercules, CA). PCR reaction was carried out using 10 μl of the sample and 15 μl of reaction mixture containing 2.5 μl of 10 X PCR buffer (Promega, Madison, WI), 1.25 units of *Taq* DNA polymerase (Promega), and 0.2 mM of each of the deoxyribonucleotides (Pharmacia LKB, Piscataway, NJ). PCR amplification was performed using a thermal cycler (Perkin-Elmer, Wellesley, MA). The bacterium-specific primer (5'-3') sequences used in this study are shown in Table 1. The primers for *P. gingivalis*, *T. forsythia*, *C. rectus*, *E. corrodens* and *T. denticola* have been described previously by Slots *et al.* [10]. The primers for *A. actinomycetemcomitans* and *P. intermedia* have been described by Ashimoto *et al.* [11], and the primer for *P. micra* was designed for this study.

PCR products were separated in a 1.5% agarose gel (Sigma, Dorset, United Kingdom) by an electrophoresis performed at 5 V/cm in Tris-acetate-EDTA buffer (Promega). The DNA bands on the gel were stained with 0.5% $\mu\text{g/ml}$ ethidium bromide (Amersham, Arlington Heights, IL) and photographed under 300-nm UV light illumination.

The frequencies of bacterial species in the dorsum of the tongue and cheek mucosa were analyzed using the Chi-square test. We also estimated the odds ratios for the presence of colonizing periodontal pathogens according to the presence of teeth in children aged 6 to 13 years. Fisher's exact test was employed to evaluate the relationship between the prevalence of two different bacteria. All tests were performed using the statistical software BioEstat 5.0 (Instituto de Desenvolvimento Sustentável Mamirauá-Belém-Pa) and Instat 3 (GraphPad Software, Inc. La Jolla, CA).

Results

A total of 80 individuals, 40 newborns aged 0–4 months (24 male and 16 female, hereinafter 'N group') and 40 children aged 6–13 years (20 male and 20 female, hereinafter 'C group') were recruited for this study (Table 2). The C group exhibited a healthy periodontal profile based on the mean values of Visible Plaque Index (VPI; 0.44 ± 0.19) and Gingival Bleeding Index (GBI; 0.03 ± 0.05). The absence of dentition ruled out both VPI and GBI evaluation in the N group; however, based on visual inspection, healthy oral mucosal condition was confirmed for all newborns, especially in the areas of gingivae, tongue and cheek.

The prevalence of bacteria detected in the samples isolated from dorsa of tongue and cheek mucosa was compared between the edentulous N group and mixed dentition C group (Figure 1). *P. intermedia* was only found in samples taken from the C group, but not the N group (Figure 1A, and B). While *P. gingivalis* was not detected in any of the samples of either group examined, the other two red complex bacteria, *T. forsythia* and *T. denticola*, as well as *A. actinomycetemcomitans*, a pathogen for localized aggressive periodontitis, were prevalent in both tongue and cheek samples at very low prevalence. The prevalence of *T. forsythia*, *T. denticola* and *A. actinomycetemcomitans* did not show any significant increase when comparing the N and C groups (Figure 1A, and B). When samples from tongue dorsa were evaluated, the prevalence of *C. rectus*, *P. intermedia*, *E. corrodens* and *P. micra* was significantly higher in the C group compared to the N group (Figure 1A). Also, among the samples isolated from cheek mucosa, *C. rectus* and *P. intermedia* detected in the C group showed significantly higher prevalence over that found in the N group, while the remaining orange complex bacteria, *E. corrodens* and *P. micra*, did not show any remarkable difference between the two groups (Figure 1B). These results demonstrated that the prevalence of orange complex bacteria found in tongue and cheek, including *C. rectus*, *P. intermedia*, *E. corrodens* and *P. micra*, preceded colonization of red complex bacteria.

In order to evaluate the possible influence of mixed dentition on the colonization of each bacterium in the oral cavity, odds ratios expressing the difference between N and C groups in terms of the prevalence of a given bacterium were calculated. More specifically, the percentile of individuals who have a given bacterium in any site of the oral cavity (N group: tongue and/or cheek; C group: subgingival sulcus, tongue and/or cheek) was used to mark the prevalence of bacteria colonizing in the oral cavity (Table 3). As shown in Figure 1, based on the prevalence of bacteria detected in tongue and cheek when making a comparison between the N and C groups, the orange complex bacteria, including *C. rectus*, *P. intermedia*, *E. corrodens* and *P. micra*, showed significantly higher odds ratios of prevalence found in the oral cavity of children aged 6–13y than newborns (Table 3). At the same time, the prevalence of *T. forsythia* found in tongue and cheek did not show any statistical difference between the two groups (Figure 1). However, when the data of subgingival samples were added, the prevalence of *T. forsythia* found in the oral cavity also showed significantly higher odds ratios in the C group compared to the N group (Table 3), suggesting that the red complex bacterium *T. forsythia*, in contrast to any of the orange complex bacteria studied, colonizes more profoundly in a subgingival site than either tongue or cheek. Of particular note, the slight increase of prevalence found for *T. denticola*, as well as *A. actinomycetemcomitans*, did not show any statistical significance as evaluated by the calculated odds ratios (Table 3).

The prevalence of bacteria in both subgingival and tongue samples isolated from the C group was compared (Figure 2), and no significant difference was found between subgingival and tongue samples in all bacteria tested, except *T. forsythia*, which showed a significantly higher prevalence in subgingival sample than cheek samples. *P. gingivalis* was not detected in any sites of any subject from either group. Although no significant difference was detected, the prevalence of bacteria found in cheek samples tended to be lower than the prevalence of the same bacteria in either subgingival or tongue samples, including *T. forsythia* (Figure 2). These results suggested that the gingival sulcus appeared to be the key site of *T. forsythia* colonization in the C group compared to the orange complex bacteria studied, which were found to colonize in any site sampled: gingival sulcus, tongue or cheek surface.

Finally, to test our hypothesis that colonization of orange complex bacteria may be associated with the initial colonization of red complex bacteria in the oral cavity, we tested co-colonization trends among different bacteria using Fisher's exact test (Table 4). Based on the calculated prevalence of bacteria colonizing in the oral cavity, we identified a trend strongly suggesting that *T. forsythia* and *T. denticola* co-colonize in the C group. Although other combinations of red-red and red-orange co-colonization were tested, neither the C nor the N group showed a comparably significant association (Table 4). This result was contrary to our expectation because we hypothesized that colonization of orange complex is associated with the initial colonization (or increased prevalence) of red complex bacteria. When site-specific prevalence was analyzed (Figure 3), statistically significant association between *T. forsythia* and *T. denticola* was only found in the subgingival sulcus, not in tongue or cheek. These results indicated that the colonization of dentition-related *T. forsythia* in the subgingival sulcus may be associated with the increased prevalence of non-dentition-related *T. denticola*.

Discussion

The results showed a distinct pattern of age-related initial colonization of the red complex bacteria *T. forsythia* and *T. denticola*. First of all, the most putatively pathogenic red complex bacterium, *P. gingivalis*, was not detected in any subjects in either of the two groups tested. *T. denticola* was found in both N and C groups at a relatively high prevalence

(N group: 32.5%; C groups: 52.5%), suggesting that the colonization of *T. denticola* was either minimally, or not related at all, with the presence of teeth. On the other hand, the colonization of *T. forsythia* appeared to require gingival sulcus because its prevalence in subgingival samples of children was significantly higher than that of tongue in children compared to *C. rectus*, *P. intermedia*, *E. corrodens* and *P. micra* for which the prevalence between subgingival and tongue samples in children showed no difference. As noted above, while both N and C groups showed the presence of *T. denticola* in the oral cavity, co-colonization of *T. forsythia* and *T. denticola* was significantly high in the subgingival sulcus of C group subjects, suggesting, in turn, that the colonization of dentition-related *T. forsythia* may, in fact, promote the colonization of non-dentition-related *T. denticola*, in the subgingival sulcus. Although the factors underlying the increased prevalence of *T. denticola* colonization by interaction with *T. forsythia* remain to be elucidated, these results do underscore a unique pattern of age-related initial colonization of these two red complex bacteria.

The detection of periodontal pathogens from *in vivo* samples is an important approach to identify specific pathogens, diagnose a particular disease process, and design a treatment plan for it [12, 13]. The oral cavity of edentulous children provides a variety of surfaces for bacterial attachment and possible colonization. These surfaces include epithelium of the cheeks, gums, and tongue. However, with the eruption of teeth, alterations in the oral microenvironment occur, especially the formation of the gingival sulcus, providing additional environmental niches favorable to the growth of several anaerobic species, e.g., red complex periodontopathogens. Indeed, Sakai *et al.* [14] found that a high percentage of Brazilian children with mixed dentition harbored at least one of the four putative periodontal pathogens (*A. actinomycetemcomitans*, *P. gingivalis*, *P. nigrescens* and *T. denticola*) in their saliva. We therefore hypothesized a correlation in which the prevalence of all three red complex bacteria would increase in relation to the presence of teeth. However, contrary to our expectation, the present study demonstrated that co-colonization of *T. forsythia* and *T. denticola* was significantly high in the subgingival sulcus of C group subjects and that the presence of *P. gingivalis* could not be identified in any site of any subject in either group studied (Table 3).

Tanner *et al.* [15] found higher frequencies of periodontal pathogens present in the dorsum of the tongue of children aged 19–36 months than in children aged 6–18 months. They reported the following prevalence of periodontal pathogens in children aged 6–18 months: *A. actinomycetemcomitans*, 30%, *P. intermedia*, 29%, *P. gingivalis*, 23%, *T. forsythia*, 11%, and *T. denticola*, 36%. Although we did not detect *A. actinomycetemcomitans*, *P. intermedia* or *P. gingivalis* in samples taken from the tongue dorsum of newborns (Figure 1), we did find *T. forsythia* and *T. denticola* at the prevalence of 7.5% and 20.0%, respectively, in the tongue samples of newborns (Table 3). The higher frequencies of periodontal pathogens detected in the study by Tanner *et al.* [15] may have resulted from the older age of their study subjects (6–18 months) in comparison to the subjects in our N group at 0–4 months. Nonetheless, the detection of *T. denticola* (36%) on the tongue samples of children aged 6–18 months in the study by Tanner *et al.* [15] corresponds to current study which detected a relatively high level of *T. denticola* in both newborns as well as children aged 6–13 years (32.5% and 52.5%, respectively) (Table 3). It is noteworthy that *T. denticola* was not detected in Japanese children aged 2–13 [16], Okada *et al.* [17], suggesting that diet or other geometrically unique factors may affect the colonization of *T. denticola* in young children.

The prevalence of *T. forsythia*, a Gram-negative, filament-shaped, strict anaerobic, non-pigmented oral bacterium, found in sub- or supragingival sulcus of children aged 2–13 was reported to range from 8 to 33% [15, 16, 17]. However, another study using saliva samples

did not find *T. forsythia* in young children [18]. Although the present study detected *T. forsythia* at low prevalence (10%, Figure 1 and Table 3) in newborns, the prevalence of this bacterium in children aged 6–13 was recorded at 52.5%, 15% and 10% in the sample of subgingival sulcus, tongue and cheek, respectively (Figure 3). Furthermore, the prevalence of *T. forsythia* detected in subgingival sample was significantly higher than tongue sample (Figure 3). In sum, these reports, including the present study, indicate that *T. forsythia* may start dwelling preferentially in the subgingival sulcus, but little or none in tongue, cheek or saliva, of young children.

P. gingivalis is considered to be a major pathogen of adult periodontitis [19, 20] and is occasionally detected in samples from the oral cavities of children [21]. We did not find this bacterium in any of the samples from any of the sites across age groups, and this was consistent with the reports by Frisken *et al.* [22] and Kimura *et al.* [16]. On the other hand, many other previous studies have reported the occurrence of *P. gingivalis* in children [15, 17, 23]. Such inconsistency in the detection of *P. gingivalis* may be explained by differences in factors related to chosen microbiological evaluation methods, levels of sensitivity of species detection, dentition phase, ethnic and social background and diets unique to the location where the study was carried out.

Orange complex bacteria, *C. rectus*, *P. intermedia*, *E. corrodens* and *P. micra*, were significantly more frequently found in the tongue sample of children with mixed dentition than newborns (Figure 1). Furthermore, based on the odds ratios values, the presence of teeth significantly increases the risk for colonization of these orange complex bacteria (Table 3). However, such interpretation of bacteria-tooth relationship may only be applied if biological and ecological differences between newborns and children aged 6–13 are solely attributed to the presence or absence of teeth (Table 3). It is plausible that differences in diet and other factors between these two age-related groups also affect the environment required for bacterial colonization in the oral cavity. Nonetheless, the elevated prevalence of these orange complex bacteria in children aged 6–13 compared to *P. gingivalis*, *A. actinomycetemcomitans* and *T. denticola*, which did not show significant increase of prevalence in this age group, suggests that the environment distinct to the oral cavity in children aged 6–13 can provide the niches required for the colonization of orange complex bacteria.

C. rectus, which belongs to the orange complex, was the most prevalent bacterium in the group with mixed dentition (97.5%), while one quarter (25%) of newborns possessed *C. rectus* (Table 3). The presence of teeth in the oral cavity seems to be the factor that increases the incidence of this bacterial colonization along with other orange complex bacteria in children's oral cavity. In accordance with our findings, the study by Okada *et al.* [17] demonstrated 100% prevalence of *C. rectus* for every age between 2 and 12 based on plaque samples from tooth brushing for one minute. Kimura *et al.* [16] also found the prevalence of *C. rectus* to be about 50% in the supragingival plaque on molars of children in Japan (2–13y old, 12 subjects in each year of age, a total 144 children). Tanner *et al.* [15] also reported that *C. rectus* could be found at a prevalence of 43% and 50% on supragingival plaque of the upper anterior right incisor of children aged 19–36 months and 6–18 months, respectively. These slight inconsistencies may have occurred as a result of the differences in age populations recruited in each study, as well as the method of plaque sampling. Nonetheless, it appears that *C. rectus* can colonize in the oral cavity at relatively high prevalence as early as newborn. It would be intriguing to investigate whether such early colonization of *C. rectus* can lead to the onset of periodontal disease, as some previous studies demonstrated that *C. rectus* can be found in the gingival sulcus of periodontally healthy subjects, suggesting that this bacterium may be commensal [24, 25].

According to Kimura et al [16], *P. intermedia* was not found at all in 2–4y healthy children and only infrequently detected (about 5–20%) in 5–13y healthy children. In the present study, this bacterium was not present in newborns, but it was found in 57.5% of the tongue samples and 75.0% of the subgingival samples of children aged 6–13 years. Furthermore, all 40 children showed positive for *P. intermedia* DNA as detected at any site sampled (Subgingival/Tongue/Cheek: Table 3). Our result is supported by Könönen et al [2] who also did not find this pathogen in a study of children ranging in age from 2 months to 2 years of age. On the other hand, Kamma et al [26] found this bacterium to be prevalent at the range of 35–42.50% in children with mixed dentition. Whether at high or low level, the elevated prevalence of *P. intermedia* in dentate children compared to newborns can be explained by tooth eruption and by *P. intermedia* being an anaerobic species which preferentially grows in the gingival sulcus.

Another orange complex bacterium, *P. micra*, has not often been investigated in studies examining bacterial colonization in young children. Kamma et al [26] reported frequencies of *P. micra* of about 12.5 – 15% in sulcus samples of children aged 7–8 years. In the present study, the prevalence of this pathogen was significantly higher on the dorsum of the tongue in children with mixed dentition compared to newborns. However, the prevalence of *P. micra* found on cheek surface did not show age-related difference (Figure 1). Having results which show significant increase of odds ratio (Table 3) and higher detection of *P. micra* in subgingival sulcus than cheek (Figure 3), it appears that *P. micra* colonizes more preferentially in gingival sulcus than cheek surface. The detection of *P. micra* might have resulted from the mechanical detachment (or release) of this bacterium from subgingival sulcus by tongue movement.

In conclusion, this study indicated that a wide range of red, as well as orange, complex periodontal pathogens can be detected in both edentulous newborns and periodontally healthy children with mixed dentition. More importantly, this investigation suggested that alterations in the oral microenvironment coincident with the eruption of teeth, especially the formation of gingival sulcus, provide environmental niches (other than tongue and cheek) favorable to the growth of periodontal pathogens, especially orange complex bacteria and *T. forsythia*, as evidenced by the prevalence of bacterial detection shown to increase in children aged 6–13y with mixed dentition compared to edentulous newborns. The development of an efficient prevention modality is the best approach to managing periodontal disease. Since our data show the likelihood of early co-colonization of red bacteria in some susceptible children under the age of 13, steps can be taken to detect and either suppress or eliminate such pathogens using an appropriate oral prophylaxis involving antibiotics.

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A) Prevalence of bacteria on the dorsa of tongue

B) Prevalence of bacteria on the cheek mucosa

Figure 1. The prevalence of bacteria detected in tongue and cheek as compared between newborn (0–4 mo) and children with mixed dentition (6–13y)

The prevalence (%) of bacteria detected by bacteria-specific DNA-PCR is shown for the samples isolated from dorsa of tongue (A) and from the surface of cheek mucosa (B) of newborns (n=40) and children aged 6–13y (n=40). The prevalence of each bacterium found in subjects of each age group was subjected to statistical analysis using a Chi square test. *, **, ***, indicate the statistical difference of prevalence between newborns and children aged 6–13y at $p<0.001$, $p<0.002$, or $p<0.02$, respectively.

Figure 2. The prevalence of bacteria detected in the subgingival sulcus, tongue and cheek samples collected from children aged 6–13y

The prevalence (%) of bacteria detected by bacteria-specific DNA-PCR is shown for the samples isolated from subgingival sulcus, dorsa of tongue and surface of cheek mucosa of children aged 6–13y (n=40). The prevalence of each bacterium found in each respective site was subjected to statistical analysis using a Fisher's exact test. *, indicates the statistical difference at $p < 0.02$.

Figure 3. Site-specific prevalence of co-colonization of *T. forsythia* and *T. denticola* detected in the subgingival sulcus, tongue and cheek samples collected from children aged 6–13y
 The prevalence (%) of *T. forsythia* and *T. denticola* detected together or alone by bacteria-specific DNA-PCR is shown for the samples isolated from subgingival sulcus, dorsa of tongue and surface of cheek mucosa of children aged 6–13y (n=40). Fisher's exact test was employed to evaluate the relationship between prevalence of two different bacteria. +*, indicates that there is a statistically significant relation between *T. forsythia* and *T. denticola* by Fisher's exact test ($p < 0.0001$).

Table 1

Bacteria-specific primers and PCR-product sizes (base pairs)

Bacteria	Primers	Base Pairs
<i>A. Actinomycetemcomitans (Aa)</i>	5'-AAACCCATCTCTGAGTTCTTCTTC3' 5'-ATGCCAACTTGACGTTAAAT3'	550
<i>P. Gingivalis (Pg)</i>	5'-AGGCAGCTTGCCATACTGCGG-3' 5'-ACTGTAGCAACTACCGATGT-3'	404
<i>P. Intermedia (Pi)</i>	5'-TTTGTGGGAGTAAAGCGG-3' 5'-TCAACATCTCTGTATCTGCGT-3'	575
<i>T. Forsythia (Tf)</i>	5'-GCGTATGTAACCTGCCCGCA-3' 5'-TGCTTCAGTGTCAGTTATACCT-3'	641
<i>C. Rectus (Cr)</i>	5'-TTTCGGAGCGTAAACTCTTTT-3' 5'-TTTCTGCAAGCAGACACTCTT-3'	598
<i>E. Corrodens (Ec)</i>	5'-CTACTAAGCAATCAAGTTGCC-3' 5'-CTAATACCGCATACGTCCTAAG-3'	688
<i>T. Denticola (Td)</i>	5'-TAAATACCGAATGTGCTCATTTACAT-3' 5'-TCAAGAAGCATTCCTCTTCTTTA-3'	316
<i>P. Micra (Pm)</i>	5'-AGTGGGATAGCCGTTGGAAA-3' 5'-GACGCGAGCCCTTCTTACAC-3'	328

Table 2

Distribution of age and gender of the study population

	N group: 0–4Mo	C group: 6–13y	Total
Male	24	20	44
Female	16	20	36
Total (average age: mean±SD)	40 (0.22±0.13) months	40 (9.33±1.99) years of age	80

Table 3

Risk of having teeth for oral colonization of periodontal pathogen

	New Born						6–13y old					
	% (To/Ch)	% (SG/To/Ch)	OR	1/OR	95% CI	p value	% (To/Ch)	% (SG/To/Ch)	OR	1/OR	95% CI	p value
<i>Cr</i>	25.0	97.5	0.009	111.1	0.001 < CI < 0.071	< 0.0001						
<i>Pg</i>	0	0	—	—	—	—						
<i>Aa</i>	2.5	15.0	0.145	6.9	0.016 < CI < 1.269	0.114						
<i>Pi</i>	0	100	0.00015	6561.7	0.000003 < CI < 0.008	< 0.0001						
<i>Tf</i>	10.0	60.0	0.074	13.5	0.022 < CI < 0.248	< 0.0001						
<i>Ec</i>	17.5	92.5	0.017	58.8	0.004 < CI < 0.072	< 0.0001						
<i>Td</i>	32.5	52.5	0.436	2.3	0.176 < CI < 1.079	0.113						
<i>Pm</i>	40.0	90.0	0.074	13.5	0.022 < CI < 0.249	< 0.0001						

SG: Subgingival sulcus sample, To: Tongue sample, Ch: Cheek mucosa sample, OR: Odds ratio, CI: Confidence interval, % (To/Ch): Prevalence of bacteria detected in any of the sample from tongue or cheek, % (SG/To/Ch): Prevalence of bacteria detected in any of the sample from subgingival sulcus, tongue or cheek, New Born (n=40), 6–13y old children (n=40)

Table 4

Relation between two bacteria colonized in oral cavity

	<i>Cr</i>	<i>Pg</i>	<i>Aa</i>	<i>Pi</i>	<i>Tf</i>	<i>Ec</i>	<i>Td</i>	<i>Pm</i>
<i>Cr</i>		-	-	-	-	-	-	-
<i>Pg</i>	-		-	-	-	-	-	-
<i>Aa</i>	-	-		-	-	-	-	-
<i>Pi</i>	-	-	-		-	-	-	-
<i>Tf</i>	-	-	-	-		-	+ [§]	-
<i>Ec</i>	-	-	-	-	-		-	-
<i>Td</i>	-	-	-	-	-	-		-
<i>Pm</i>	-	-	-	-	-	-	-	

New Born
 6-13 y

[§] there is an association between indicated two bacteria by Fisher's exact test, $p < 0.005$.