The Relationship of *Prevotella intermedia, Prevotella nigrescens* and *Prevotella melaninogenica* in the Supragingival Plaque of Children, Caries and Oral Malodor

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Purpose: *A relationship between the distribution of periodontal bacteria species and malodor in children has not been sufficiently investigated. The present study was undertaken to determine the presence of 3 periodontopathic bacteria (Prevotella spp. P. intermedia, P. nigrescens, P. melaninogenica) in the supragingival plaques of 3 to 16-year-old children with different oral health conditions and oral malodor.* ***Methods:*** *The number of decayed and filled primary teeth (df) and Decayed, Missing and Filled permanent teeth (DMF), Papillary Marginal and Attached gingivitis (PMA) index, Oral Hygiene Index (OHI), and oral malodor of each subject were determined prior to the collection of supragingival plaques. Three periodontopathic bacteria (P. intermedia, P. nigrescens, P. melaninogenica) in supragingival plaques were detected by using an immunoslot blot assay with monoclonal antibodies specific for each microorganism.* ***Findings:*** *The frequencies of periodontopathic bacteria in children with and without caries were not significantly different from each other. Positivity for P. intermedia, but not for P. nigrescens or P. melaninogenica was correlated with oral malodor. Oral malodor was also correlated with the debris index, a component of OHI.*

The group with the higher OHI showed a higher prevalence of periodontopathic bacteria. For the 3 periodontopathic bacteria in the subjects tested, plaques positive for any of them were not age related. However, the frequencies of all 3 periodontopathic bacteria were the highest in the 3-6-year olds. ***Conclusion:*** *The supragingival plaques in children can harbor 3 species of periodontopathic bacteria, P. intermedia, P. nigrescens, and P. melaninogenica.*

Keywords: *Children, Periodontopathic bacteria, Supragingival plaque, Oral malodor*
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INTRODUCTION

Prevotella intermedia has been implicated as a pathogen in adult periodontitis,[1-2] in acute necrotizing ulcerative gingivitis,[3] and in pregnancy-related gingivitis.[4] However, it is detectable in periodontally healthy adults[5] and edentulous children as well.[6] *Prevotella nigrescens* has also been detected in the oral cavity of periodontally healthy adults and children.[7-9] Also, *Prevotella melaninogenica* has been detected in children and adult periodontal patients.[10-11] We previously reported a relationship between the frequency of *Porphyromonas gingivalis* or *Prevotella* spp. in supra or subgingival plaques and various periodontal clinical parameters according to subject age.[11] These findings suggested that the increase in probing depth with increasing age was not affected by the occurrence of *Porphyromonas gingivalis,* together with *Prevotella intermedia* and *Prevotella nigrescens* in subgingival plaque of the adult age groups, bacteria which might be responsible for the high frequency of periodontal disease in the older age groups. They suggested that the supragingival plaque in 6-to 9-year-old children probably harbor *Porphyromonas gingivalis.*

Ximenez-Fyvie *et al*.[12] reported that the microbial composition of supra and subgingival plaque can harbor putative periodontal pathogens, suggesting a possible role of the environment as a reservoir of such species for the spread or reinfection of subgingival sites. Also, some investigators

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previously reported the finding of periodontopathic bacteria in supragingival plaques of adults and children.[11-16] However, few comprehensive studies of the microbial composition of supragingival plaques of children and oral malodor have been carried out. In addition, the role of supragingival plaques in the initiation of gingivitis and/or periodontal diseases in children is unknown. We previously reported that the supragingival plaques in children can harbor periodontopathic bacteria such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetem comitans,*[15] even in the absence of caries.

Umeda *et al*.[17] recently reported that high plaque retention seems to promote the colonization of periodontal pathogens in the oral cavity of children and that *Prevotella intermedia* and *Prevotella nigrescens* were detected more frequently in those children.

The aim of the present study was to examine the supragingival plaque of children with different oral health conditions for the presence of 3 species of periodontopathic bacteria of the genus *Prevotella*, i.e., *P. intermedia*, *P. nigrescens*, and *P. melaninogenica* and also to investigate the relationship between these bacteria and oral malodor.

MATERIALS AND METHODS

Subjects and clinical parameters

Sixty children (males: 37, females: 23, age range: 3.0 to 16.0 years-old) with or without active caries (open caries) at the Meikai University Hospital in Saitama Prefecture, Japan were examined. Clinical parameters such as df and DMF, PMA index,[18] OHI,[19] and oral malodor were determined prior to the collection of supragingival plaque. In addition, oral malodor was determined as the quantity of intraoral ammonia measured using the Atein™ (Mitoleben Co, Japan).[20] The exclusion criteria included antibiotic therapy in the previous 5 months and any systemic conditions that could influence the course of periodontal disease or would require premedication. Informed consent was obtained from each parents/guardians. Ethical clearance for the study was obtained from the local ethics committee.

Sample collection

Supragingival plaque from each subject was obtained from primary or permanent first molars with or without active caries using an excavator. Then one loop-full (Catalog no. 254410; Nunk Co., Copenhagen, Denmark) of collected plaque was transferred to a microtube containing 500 μl of sodium carbonate buffer (pH 9.6). The collected plaque samples from each subject were pooled together at –80ºC and then thawed immediately before being analyzed by using an immunoslot blot assay. The plaque samples were dispersed by pulsed sonication (Sonifier cell disrupter; Branson Sonic Power Co., Danbury, Conn.) for 10 s at 40 W. The dispersed samples (25μl) were used for the immunoslot blot assay.[10]

Bacterial strains

P. intermedia ATCC 25611, *P. nigrescens* 25261, and *P. melaninogenica* 25845 were used in this study.

Monoclonal antibodies

The following monoclonal antibodies specific for their respective bacteria were used[10, 21]: Monoclonal antibody BIF6 antibody against *P. intermedia*, BIF5 antibody against *P. nigrescens* and BMF4 antibody against *P. melaninogenica*.

Immunoslot blot assay: This assay was performed by using the procedure previously described.[10] Briefly, supragingival plaque samples (25 μl) suspended in 500 μl sodium carbonate buffer or each sonicated bacterial extract (25 ng of protein) was blotted onto nitrocellulose paper (Bio-RaD: Tokyo, Japan) in each well of an immunoslot blot apparatus (Hybrislot manifold: Bethesda Research Laboratories, Gaithersburg, MD). After blotting, the paper was treated for 30 min with 3% gelatin and washed with phosphate-buffered saline containing 0.02% (vol/vol) Tween 20 (PBS-Tween). After this wash, the paper was treated for 60 min with culture supernatants of monoclonal antibody producing cells or of control SP2/O- Ag 14 myeloma cells and then washed by shaking for 30 min with PBS-Tween. The treated paper was thenincubated for 60 min with a horseradish peroxidase-con-

Table 1. Median values and range of clinical parameters in children with and without active caries.

Clinical parameter	with caries (n=27)	without caries (n=33)	Median test
df tooth number	10.0 (0-19)	5.0 (0-12)	**
DMF tooth number	4.0 (0-23)	3.0 (0-14)	
PMA index	2.0 (0-8)	1.0 (0-7)	
Debris index	2.2 (0.7-3.0)	2.0 (0.3-3.2)	
Calculus index	0 (0-0.2)	0 (0-0.17)	
Oral hygeine index	2.2 (0.7-3.0)	2.0 (0.3-3.2)	
Oral malodor	28.0 (2-60)	25.0 (3-82)	*

Median test : with caries vs without caries, * $p<0.05$, ** $p<0.01$.

Table 2. Median values of range of clinical parameters of BIF6 and BIF5 positive or negative reactivity in children with and without active caries

	Clinical parameter	Positive reactivity	Negative reactivity	Median test
BIF6	Debris index	2.17 (Range: 2-3.2)	1.67 (Range: 0.3-3.0)	*
	Oral hygiene index	2.17 (Range: 2-3.2)	1.67 (Range: 0.3-2.5)	*
BIF5	df tooth number	10.0 (Range 3-19)	3.0 (Range 0-14)	*
	DMF tooth number	6.0 (Range 0-23)	0.0 (Range 0-4)	**

Median test :positive reactivity vs negative reactivity, *p<0.05 **p<0.01

Table 3. Distribution of 3 periodontopathic bacteria in cildren with and without active caries

	No. positive subject/sampled subjects (%)		
Periodontopathic bacteria	with caries (n=27)	without caries (n=33)	total (n=60)
Prevotella intermedia	5/27 (18.5)	5/33 (15.2)	10/60 (16.7)
Prevotella nigrescens	7/27 (25.9)	7/33 (21.2)	14/60 (23.3)
Prevotella melaninogenica	7/27 (25.9)	6/33 (18.2)	13/60 (21.7)

jugated goat anti-mouse immunoglobulin G (Bio-Rad) washed, and then the immunocomplexes were visualized by using horseradish peroxidase color development reagent (Bio-Rad). Densitometric analysis of the reactivities was performed with a graphic analyzer (Shoni GA: Showa Denkoh, Tokyo, Japan). The reactivity was evaluated as positive when the intensity of a test sample was more than that assigned to 1×10^6 cells of each periodontopathic species, according to our previous investigation[11] which examined the degree of colonization and reactivity of monoclonal antibody specific for each periodontopathic bacterium. The results were expressed as the percentage of plaque samples that reacted with the species-specific monoclonal antibodies.

Statistical analysis

Clinical parameters were analyzed using the General Linear Models and ANOVA for multiple comparisons.

The prevalence of reactivity with the 3 monoclonal antibodies was analyzed using Chi-Square Test.

RESULTS

The median values and range of each clinical parameter for subjects with and without active caries are shown in Table 1. As expected, a significant difference between children with and without active caries was observed in terms of the df index (Median test, $p<0.01$) and oral malodor (Median test, $p<0.05$). However, there were no significant differences between children with and without active caries for any other clinical parameter. The median values for clinical the parameters of *P. intermedia* and *P. nigrescens* positive or negative subjects with and without active caries are shown in Table 2. A significant difference between children with and without active caries was observed in terms of the debris index and oral hygiene index in *P. intermedia*-positive or -negative subjects (Median test, $p<0.05$, $p<0.01$) and also in terms of the df and DMF indexes in *P. nigrescens*-positive or -negative ones (Median test , $p<0.05$,$p<0.01$).

The distribution of *P. intermedia*, *P. nigrescens*, and *P. melaninogenica* in supragingival plaque of children with and without active caries is shown in Table 3. The prevalence of

Table 4. Percentage distribution of periodontopathic bacteria in children by age

	Age (years)													
Periodontopathic bacteria	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Prevotella intermedia	66	25	10	40	0	0	11	0	0	100	14	14	0	0
Prevotella nigrescens	66	0	20	40	0	20	11	0	0	100	43	29	0	0
Prevotella melaninogenica	33	0	30	40	0	20	11	0	100	0	43	29	0	0
No. of subjects	3	4	10	5	5	5	9	2	1	1	7	7	0	1

plaque positive for the 3 periodontopathic bacteria in the two subject groups tested was significantly different. The prevalence of *P. nigrescens* and *P. melaninogenica* in children with caries was the same (25.9%). The prevalence of *P. intermedia*, *P. nigrescens* and *P. melaninogenica* in subjects with caries was greater than that in those without caries.

The percent distribution of the 3 species in children by age is summarized in Table 4. All 3 periodontopathic bacteria were detected in most 3-6-year-old children, with the frequency being lower in the 7-12-year-old and the 13-16-year-old. The number of periodontopathic bacteria positive subjects *per* site was greater in the 3-6 and 13-16 age groups.

The minimum age of subjects positive for *P. intermedia* and *P. nigrescens* was 3 years and 5 months, whereas that for *P. melaninogenica* was 3 years and 11 months. The percent of the subjects (%) positive for *P.intermedia*, *P. nigrescens*, and *P. melaninogenica* in relation to the 3-6, 7-12 and 13-16 age groups, corresponding to the stages of dental development is shown in Figure 1. No significant difference between age groups was found, possibly due to the small number of positive-subjects in the groups.

Afterwards, we examined whether these periodontopathic bacteria were related to oral malodor. We found that the median values of oral malodor for subjects positive for *P. intermedia, P. nigrescens* and *P. melaninogenica* were 25.0 (range: 10.0-43.0), 30.0 (range: 2.0-48.0) and 26.0 (range: 2.0-50.0), respectively. For the subjects, negative for *P. intermedia*, *P. nigrescens*, and *P. melaninogenica*, these values were 26.0 (range: 3.0-35.0), 28.0 (range: 16.0-55.0), and 27.0 (range: 8.0-60.0), respectively.

There was no significant difference between the two groups. However, oral malodor in subjects positive for *P. intermedia*, *P. nigrescens*, and *P. melaninogenica* was correlated with the debris index (r^2=0.354, $p<0.05$; Figure 2), suggesting that the debris in the oral cavity was responsible for the oral malodor.

DISCUSSION

We reported previously that periodontopathic bacteria such as *P. gingivalis* could be detected in the supragingival plaque of children with and without caries and that these periodontopathic bacteria were found at an early age.[15] These findings are consistent with those of Tanner *et al*.[22] Also, we reported a relationship between the frequency of immunoreactivity for *Porphyromonas gingivalis* and *Prevotella* spp. in supra- or subgingival plaques, and periodontal clinical parameters according to subject age. This finding suggested that the supragingival plaque of 6– to 9-year-old children harbor *Porphyromonas gingivalis.*[11] This present study did not show the alteration of the prevalence of *P. intermedia*, *P. nigrescens* and *P. melaninogenica*.. Together the present finding and our previous findings,[11] lead us to conclude that supragingival plaque in children preferentially harbor *P. gingivalis* than *P. intermedia*, *P. nigrescens* and *P. melaninogenica* under healthy conditions.[15] In addition, we recently reported that *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans,* particularly *P. gingivalis* in the oral cavity in children were distributed in plaque taken from teeth, tongue and buccal mucosa, suggesting that also the tongue and the buccal mucosa may be a potential microbial reservoir for tooth-associated species and that periodontopathic bacteria may be considerably enhanced in circumpubertal children.[23] We found in the present study that *P. intermedia*, *P nigrescens*, *and P. melaninogenica* were detected in the supragingival plaque of children and also that these periodontopathic bacteria were detectable over a broader age range, being found at an earlier age, 3 to 6 year old children. However. no difference in prevalence was found among the age groups (Figure 1). This finding differs greatly from the prevalence regarding *P. gingivalis* [11]

It may be necessary to continue to monitor individuals who are positive for these periodontopathic bacteria, since their risk of developing periodontal disease may be increased in the future. A longitudinal investigation would

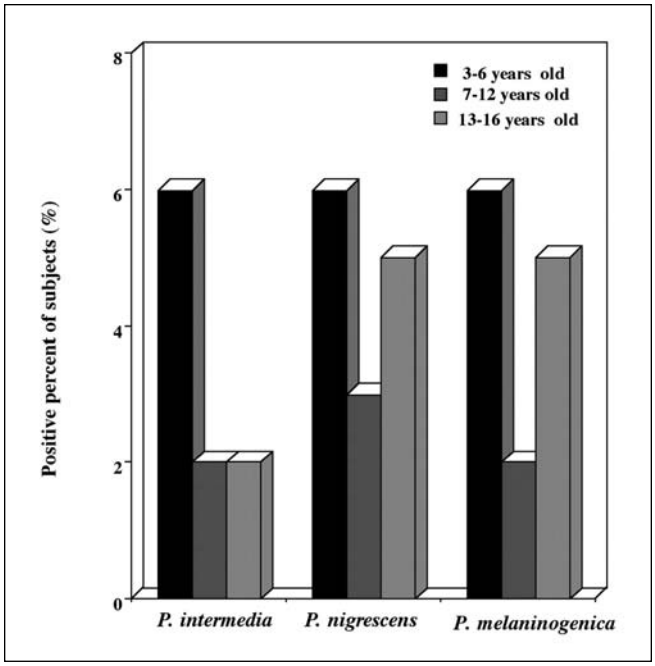

Figure 1. Prevalence (% positive) of periodontopathic bacteria for each age group. No significant difference in the prevalence of periodontopathic bacteria between the age groups was observed.

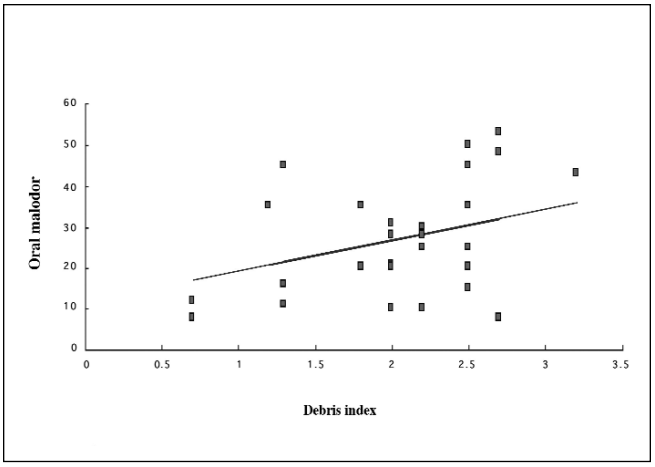

Figure 2. Comparison of oral malodor and debris index for subjects positive for P. intermedia, P. nigrescens, and P. melaninogenica. The regression line for oral malodor vs. debris index is shown. (r2=0.354 , p<0.05).

be particularly important to monitor these bacteria during the transition from periodontal health to disease.

Matto *et al*.[8] reported the distribution of oral *P. intermedia* and *P. nigrescens* in various periodontal status groups. They studied periodontally healthy and diseased individuals by using hybridization with no radioactively labeled species-specific oligonucleotide and found that *P. intermedia* and *P. nigrescens* may occur simultaneously in the oral cavity; the colonization was stable and *P. intermedia* was associated with periodontal disease. In addition, they strongly emphasized that *P. nigrescens* was detected in healthy children. Umeda *et al.*[17] recently investigated the distribution of periodontopathic bacteria in the oral cavityof children and their parents by PCR detection as well as the relationship between the bacterial findings and clinical parameters. They indicated that high plaque retention seemed to promote the colonization of periodontal pathogens in the oral cavities of children and that *P. intermedia* and *P. nigrescens* were detected more frequently in the oral cavity of children whose parents already harbored these bacteria, suggesting familial transmission. Also, using the PCR assay Kimura *et al.*[24] previously assessed the prevalence by age of 10 putative periodontopathic microorganisms in periodontally healthy children and found *P. intermedia* to be detected less frequently. In contrast, the percentage of *P. nigrescens*-positive subjects in the primary dentition increased with age, and reached about 50% at 7 years of age and older. Thus, colonization by many putative periodontopathic microorganisms can occur quite early in childhood without clinical signs of periodontal disease.

Oral malodor is a common condition affecting people of all ages. However, there are few studies about oral malodor in children.[15, 20, 25-26] We previously indicated that oral malodor in children was significantly correlated to the debris index, a component of the OHI.[15] Yosida *et al*.[20] reported that children with noticeable halitosis had higher average concentrations of ammonia in air samples taken from their mouth than did those without halitosis. Patients with periodontal disease frequently suffer from oral malodor,[27-31] and positive correlations have been demonstrated between the severity of periodontitis and the levels of volatile sulfur compounds in mouth air.[27, 32] Figueiredo *et al*.[28] previously researched whether there is some relationship between the presence of N-benzoyl-DL-arginine-2-napthylamide (BANA)-hydrolyzing species such as *Treponema denticola*, *Porphyromonas gingivalis*, and *Bacteroides forsythus* (in dental plaque, tongue scrapings, and saliva) and clinical parameters in patients with or without periodontal disease. They demonstrated that the BANA hydrolyzing bacteria in subgingival plaque are an important source of oral malodor production in the oral cavity. Ratcliff and Johnson[29] demonstrated a relationship between oral malodor and gingivitis or periodontitis, and emphasized the potential importance of volatile sulfur compounds in the transition of periodontal tissues from clinical health to gingivitis and then to periodontitis. Recently, many studies on oral malodor in children have been conducted.[20,25-26] However, clinical approaches concerning halitosis in children may be lacking. Experimental evidence strongly suggests that putrefaction of sulfur-containing proteinaceous substrates by predominantly Gram-negative oral microorganisms such as *Fusobacterium* species and *Bacteroides* species, is a primary cause of oral malodor.[33-34] Faryavi-Gholami *et al.*[26] reported on oral malodor in children and volatile sulfur compound-producing bacteria in saliva. They demonstrated that children with parent-perceived oral malodor exhibited significantly higher concentrations of odontogenic bacteria in their saliva than those without parent-perceived malodor . Additionally the levels of *Prevotella oralis* were significantly higher in children with oral malodor.

In the present study, oral malodor in subjects positive for *Prevotella* spp. *P. intermedia*, *P. nigrescens*, and *P. melaninogenica* was correlated with the debris index (Figure 2). This finding clinically suggests that tooth brushing plays a crucial role in preventing not only caries, gingivitis, and periodontitis in children, but also oral malodor due to *Prevotella* spp. as well as *P. gingivalis*.

CONCLUSIONS

P. intermedia, *P. nigrescens*, and *P. melaninogenica* were detected in the supragingival plaque of children with and without active caries. Oral malodor of subjects positive for *P. intermedia*, *P. nigrescens*, and *P. melaninogenica* was significantly correlated with the debris index, a component of the OHI.

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