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Colonization Pattern of Periodontal Bacteria in Japanese Children and Mothers

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Running title: Periodontal bacteria in children
Keyword: Transmission, Child, Colonization, Periodontal microbiota

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**Background and Objective:** The purpose of this study was to determine time of infection by anaerobic Gram-negative rods associated with periodontal disease, and clarify their transmission from mother to child.

**Method:** Seventy-eight Japanese children, including 10 siblings, aged from 3 to 9 years, and 68 mothers were enrolled in this study. Colonization by 11 periodontal bacterial species was determined using polymerase chain reaction (PCR) in samples of subgingival plaque obtained from the children and their mothers.

**Results:** Detection rates of *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola* increased after the age of 6 years. We found a high consistency in colonization by *P. gingivalis*, *T. denticola*, *Prevotella intermedia* and *Prevotella nigrescens* in 9 of the 10 siblings. The average number of bacterial species in plaque samples harboring *Fusobacterium nucleatum* and/or *Fusobacterium periodonticum* was significantly greater than that in those without, in both children and mothers. Kappa statistical analysis revealed that detection of *Capnocytophaga gingivalis*, *Capnocytophaga ochracea*, *Campylobacter rectus* and *T. denticola* in children was consistent with that in the mother.

**Conclusion:** Periodontal bacterial colonization in Japanese children increased with age and was associated with *F. nucleatum* and/or *periodonticum*, and bacterial flora in
children was similar to that in their mothers.
Introduction

More than 500 taxa of microorganisms have been identified in human oral cavity biofilm, among which, an increasing number of anaerobic Gram-negative rods and spirochetes have been demonstrated to be closely associated with various types of periodontal disease (1, 2). The predominant form of periodontal disease in children is gingivitis, initially a reversible inflammatory reaction in marginal gingival tissue. Several reports have suggested the involvement of specific anaerobic bacteria in the etiology of gingivitis in children (3, 4). Anaerobic bacteria constitute a significant portion of the bacterial community in periodontal lesions (5). Their ability to adhere and survive by evading host defenses in the rapidly changing environment of early childhood is a fundamental factor in the organization of periodontopathic biofilm (6, 7).

Many research groups have demonstrated that colonization by periodontal bacteria is a key step in the development of periodontal disease (8-10). Although many research groups have revealed a relationship between specific pathogens in lesions in children and gingivitis (11-14), relatively little is known about when such colonization takes place or the succession of such anaerobic bacterial species. Therefore, the detection of a specific bacterium in association with periodontal lesions may prove an important tool in the diagnosis and treatment of periodontal disease (15-17).
A number of studies have suggested intrafamilial infection by periodontopathic bacteria (18-20). It is possible that periodontopathic bacteria are transmitted from mother to child as the first step in colonization. Umeda et al. (21) detected *Tannerella forsythensis*, *P. intermedia* and *Prevotella nigrescens* more frequently in the oral cavities of children whose parents already harbored those bacteria, leading them to suggest intrafamilial transmission. Tanner et al. (22) found a similarity between the oral microbiota of pre-school children and that of their caregivers. These reports suggested vertical transmission of periodontopathic bacteria. Further analysis is required, however, to determine route and period of infection. Children with dental plaque harboring bacteria showed no manifest signs of gingivitis (4). Clarification of these points may offer a strong tool in the prognosis of periodontal conditions. The goal of the present study was to investigate colonization by 11 species of periodontal bacteria, including *Fusobacterium nucleatum* and/or *Fusobacterium periodonticum*, in children by polymerase chain reaction (PCR), and determine whether there was a relationship between the presence of such bacteria in children and their presence in the mothers to clarify infection route and period.

**Materials and methods**
Subjects

A total of 78 patients, including 10 siblings aged from 3 to 9 years, who visited the Department of Pediatric Dentistry, Tokyo Dental College, Chiba, Japan, and 68 mothers were enrolled in this study. None had either moderate or severe gingivitis. No patient had received antibiotics for a period of 6 months prior to the experiment. Informed consent was obtained from many of the children and all of the mothers. This study was performed with the permission of the Ethical Committee of Tokyo Dental College.

Sampling of subgingival plaque

Collection of subgingival plaque was performed according to the method of Nakagawa et al. (12), with minor modification. Briefly, in the children, subgingival plaque was collected from the maxillary first deciduous molar and maxillary second deciduous molar with sterile toothpicks after removal of supragingival dental plaque. In the mothers, samples were collected from the maxillary first molar and maxillary second molar by the same method. The collected plaque samples from each subject were suspended and mixed in 100 µl phosphate buffered saline (PBS, pH 7.2), and the microorganisms were precipitated by centrifugation at 18,870 g at 4°C for 10 min. The
pellets were then stored at -20°C until use for detection of periodontal bacteria.

**Detection of periodontal bacteria by polymerase chain reaction (PCR)**

Collected samples were suspended in 100 µl buffer consisting of 20 mM Tris-HCl pH 8.0, 2 mM EDTA and 1% triton X-100, and boiled at 100°C for 10 min. Genomic DNA was isolated by phenol extraction and ethanol precipitation. The presence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *P. intermedia*, *P. nigrescens*, *Treponema denticola*, *T. forsythensis*, *Capnocytophaga sputigena*, *Capnocytophaga ochracea*, *Capnocytophaga gingivalis*, *Campylobacter rectus* and *F. nucleatum/periodonticum* was determined by PCR. The specific primers for the PCR are listed in Table 1. All primers, except those for *F. nucleatum/periodonticum*, were designed by Ashimoto and Hayashi (14, 23). The primers for *F. nucleatum/periodonticum* were designed based on the 16S and 23S rRNA sequences of *F. nucleatum* in GeneBank at the National Center of Biotechnology Information. The specificity of the primers was confirmed against 39 oral bacterial species. Two µl sample was added to 48 µl buffer (TAKARA BIO Inc., Shiga, Japan) containing 0.2 mM dNTP, 1 µM each specific primer pair and 0.25 U Taq DNA polymerase (TAKARA BIO Inc.). All PCR runs, apart from those for *F. nucleatum/periodonticum*, were
performed using a thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, Foster City, CA) according to the method of Ashimoto and Conrads (23, 24). PCR for *F. nucleatum/periodonticum* was performed as follows: 94°C for 5 min, 30 cycles of 98°F for 15 s, 65°C for 30 s and 74°C for 30 s. The PCR products were electrophoresed using 2% agarose gel and then examined under ultraviolet light after staining with SYBR Safe DNA stain (Molecular Probe, Eugene, OR).

**Statistical analysis**

To investigate the relationship between age and detection, the Fisher exact test was performed. To determine degree of agreement, the Kappa statistic was used according to the method of Cohen (25). To clarify the relationship between colonization by *F. nucleatum/periodonticum* and detection number of other bacterial species, the Student’s *t* test was used.

**Results**

**Specificity of primers for *F. nucleatum/periodonticum***

To confirm the specificity of the primers for *F. nucleatum/periodonticum*, 39 species of microorganisms were subjected to PCR reaction. Only *F. nucleatum/periodonticum* were amplified.

**Age and detection of periodontopathic bacteria***
The detection rates for the targeted 11 species of Gram-negative bacteria in the plaque samples from the 78 children are shown in Fig. 1. Detection rates of the 3 Capnocytophaga species were relatively high compared with those of *P. gingivalis*, *T. forsythensis* and *T. denticola*. Apart from the 3 Capnocytophaga species, the detection rates of most of the other species increased with age. Detection rates of *P. gingivalis*, *T. forsythensis* and *T. denticola* increased from age 6-7 years onward. The Fisher exact test revealed a significant difference between the group of children in which *P. gingivalis* was detected by the age of 3-5 years and the 6-9-year-old group (p<0.05).

**Relationship between colonization by *F. nucleatum/periodonticum* and number of detected bacterial species**

The average detection numbers of bacterial species in relation to *F. nucleatum/periodonticum* in children and their mothers are summarized in Table 2. We found that the number of detected bacterial species in children harboring *F. nucleatum/periodonticum* was significantly higher than that in children who did not (p<0.001). The average number of bacterial species in mothers in whom *F. nucleatum/periodonticum* was detected was significantly higher than that in mothers who did not harbor *F. nucleatum/periodonticum* (p<0.001).
**Detection rate between mother and child**

The detection rates in the children and their mothers, and in the children and mothers simultaneously, for all species targeted are shown in Fig. 2. The detection rates for *C. sputigena, C. gingivalis, C. ochracea, C. rectus, F. nucleatum/periodonticum, P. nigrescens, A. actinomycetemcomitans, P. intermedia, T. denticola, T. forsythensis* and *P. gingivalis* in children were 71.8%, 50.0%, 35.9%, 42.3%, 29.4%, 38.5%, 9.0%, 19.2%, 19.2%, 10.2% and 9.0%, respectively; and those in the mothers were 80.9%, 63.2%, 39.7%, 51.5%, 39.7%, 57.6%, 23.5%, 26.5%, 39.7%, 14.7% and 17.6%, respectively.

The rates of *C. sputigena, C. gingivalis, C. ochracea, C. rectus, F. nucleatum/F. periodonticum, P. nigrescens, A. actinomycetemcomitans, P. intermedia, T. denticola, T. forsythensis* and *P. gingivalis* detected in children and their mothers simultaneously were 55.1%, 43.6%, 26.9%, 33.3%, 16.7%, 26.9%, 3.8%, 9.0%, 15.4%, 2.6%, and 2.6%, respectively.

**Consistency of detection in families**

Kappa statistic analysis revealed that detection of *C. gingivalis* (κ=0.43), *C. ochracea* (κ=0.51), *C. rectus* (κ=0.50) and *T. denticola* (κ=0.46) in children was highly consistent with that in their mothers (Table 3). It also showed an extremely high consistency of detection, or non-detection, for *P. gingivalis* (κ=1), *T. denticola* (κ=1), *P.
intermedia (κ=0.88) and P. nigrescens (κ=1) in siblings (Table 4).

Discussion

Among the 11 periodontal bacteria targeted, the detection rates of C. sputigena, C. gingivalis and P. nigrescens were higher than those of the red complex in the dental plaque samples from the children. C. sputigena, C. ochracea and C. gingivalis were reported to be highly prevalent in Japanese children (11, 14). In the present study, C. rectus was detected in 42.3%. Hayashi et al. (26) reported that all subjects were positive for C. rectus in 10 children aged 4–6 years with complete primary dentition, and that the rate of positive sites was 17.6% ± 2.4. The high detection rate of C. rectus in this study agrees with the result of their report. In this study, P. nigrescens was detected in 38.5% of the children. Umeda et al. (21) reported a similar detection rate (42.9%). Taken together, these results suggest that the 3 Capnocytophaga species, C. rectus and P. nigrescens become established at an early age in Japanese children.

We detected P. gingivalis, T. forsythensis and T. denticola in 9.0%, 10.2% and 19.2%, respectively. These species are known as the red complex, and are believed to be intimately associated with chronic periodontitis (27). McClellan et al.
(28) detected \textit{P. gingivalis} in 37.0\% of 198 subjects aged from 0-18 years old in Ohio State, USA. Umeda et al. (21) reported the detection rate of the three species to be 8.9-48.2\% in children. On the other hand, Kimura et al. (29) reported no detection of \textit{P. gingivalis} or \textit{T. denticola} in dental plaque samples from 144 Japanese children with negligible periodontal inflammation. The detection rates of these species in the present study were within a similar range to that found in previous studies.

The detection rate of \textit{A. actinomycetemcomitans} was 0.9\% in 78 children with no definite gingivitis in this study. Okada et al. (30) reported that the percentages of \textit{A. actinomycetemcomitans} in healthy, gingivitis and periodontitis groups in 2-12 year-old Japanese children were 4.8\%, 6.8\% and 20.0\%, respectively. On the other hand, Kimura et al. (29) reported that \textit{A. actinomycetemcomitans} was found in approximately 50\% of dental plaque samples collected with a Gracy curette from all age groups in 2- to 13-year-old children. \textit{A. actinomycetemcomitans} has several pathogenic factors such as fimbriae and leukotokin (31-33). These factors play an important role in colonization by these species. It is possible that the difference in detection rates between this and the study of Kimura et al. (29) are a result of genetic variation in these factors or differences in method of dental plaque sampling.

We found that the detection rates of the red complex periodontal pathogens
were higher after eruption of the permanent teeth, and that, among them, detection of *P. gingivalis* was significant (*p*<0.05). We detected all 3 species of the red complex in the dental plaque sample from one child, and *T. forsythensis* and *T. denticola* in another aged 8 years. The detection rates of these species, especially those of *P. gingivalis* and *T. forsythensis*, increased after the age of 7 years. The present results suggest that the eruption of a permanent tooth is involved in colonization by these species.

The detection rate of *F. nucleatum/periodonticum* was 32.1% in all 78 children, and the average number of periodontal bacteria species in subjects with these species was significantly higher than that in those without. Bradshaw et al (34), Foster and Kolenblander (35) and Edwards et al (36) showed that the presence of *F. nucleatum* in dental plaque played a significant role in biofilm formation by co-aggregation with other periodontal bacteria. Growth support of *P. gingivalis* by *F. nucleatum* under oxygenated and carbon-dioxide-depleted environments was reported (37). It is possible that colonization by *F. nucleatum/periodonticum* triggers periodontal bacterial colonization.

Detection rate profiles were similar between mother and child. Kappa statistical analysis revealed that detection of 2 of the Capnocytophaga species and *T. denticola* in children was consistent with detection in the mother. Notably, the
detection rate of *T. denticola* in both mother and child was only 16.4%. In addition, highly consistent detection of *P. gingivalis, T. denticola, P. intermedia* and *P. nigrescens* was found in the 10 sibling children, although the overall detection rate of these microorganisms in children was less than 19.2%. The similarity in detection rate profiles between mother and child and the consistency in detection of the species with low detection rate between family members suggests intra-familial transmission of periodontopathic bacteria. Many research groups, including our department, have demonstrated intra-familial transmission of periodontal bacteria (18-20, 38-40). Tanner et al. (22) reported that there were significant positive associations in species detection between caregiver and child. Lee et al. (41) investigated the transmission of red complex species by BANA test and reported that, if the caregiver was BANA-positive, the odds of the child also being BANA-positive were 35 times higher than for a child with a BANA-negative caregiver, after adjustment for the child's age and papillary bleeding score. In an earlier study, *P. gingivalis* infection from spouse was observed in 6 out of 16 couples (19). Taken together, our results and those of these earlier reports suggest that intra-familial transmission of periodontopathic bacteria is an important factor in the organization of periodontopathic dental plaque biofilm. To clarify this process, further study on the intra-familial transmission of these
periodontal bacteria using pulsed field electrophoresis is required.

Acknowledgements

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References


28. McClellan DL, Griffen AL, Leys EJ. Age and prevalence of *Porphyromonas*


41. Lee Y, Straffon LH, Welch KB, Loesch WJ. The transmission of anaerobic
Table 1. Species-specific and ubiquitous PCR primers for 11 periodontal bacteria

<table>
<thead>
<tr>
<th>Primer pairs (5'-3')</th>
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<td><strong>A. actinomycetemcomitans</strong></td>
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</tr>
<tr>
<td>AAA CCC ATC TCT GAG TTC TTC TTC</td>
<td>557</td>
</tr>
<tr>
<td>ATG CCA ACT TGA CGT TAA AT</td>
<td></td>
</tr>
<tr>
<td><strong>P. gingivalis</strong></td>
<td></td>
</tr>
<tr>
<td>AGG CGA CTT GCC ATA CTG CG</td>
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<tr>
<td><strong>P. intermedia</strong></td>
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<td>TTT GTT GGG GAG TAA AGC GGG</td>
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</tr>
<tr>
<td>TCA ACA TCT CTG TGG GCT GCG T</td>
<td></td>
</tr>
<tr>
<td><strong>P. nigrescens</strong></td>
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</tr>
<tr>
<td>ATG AAA CAA AGG TTT TCC GGT AAG</td>
<td>804</td>
</tr>
<tr>
<td>CCC ACG TCT CTG TGG GCT GCG A</td>
<td></td>
</tr>
<tr>
<td><strong>T. denticola</strong></td>
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</tr>
<tr>
<td>TAA TAC CGA AGC TCA TTT ACA T TCA AAG TCT CTG</td>
<td>316</td>
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<tr>
<td>TGG GCT GCG A</td>
<td></td>
</tr>
<tr>
<td><strong>T. forsythensis</strong></td>
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GCG TAT GTA ACC TGC CCG CA 641
TGC TTC AGT GTG AGT TAT ACC T
C. sputigena
AGA GTT TGA TCC TGG CTC AG 185
GAT GCC GCT CCT ATA TAC CAT TAG G
C. ochracea
AGA GTT TGA TCC TGG CTC AG 185
GAT GCC GCT CCT ATA TAC TAT GGG G
C. gingivalis
AGA GTT TGA TCC TGG CTC AG 185
GGA CGC ATG CCC ATC TTT CAC CAC CGC
C. rectus
TTT CGG AGC GTA AAC TCC TTT TC 227
TTT CTG CAA GCA GAC ACT CTT
F. nucleatum/periodonticum
CTG AAC ATT GGA AAC TAT ATA GTA GAA CAA ACA AG 142
GTC CTT CAT CGG CTC TTA CTA CCT AGG C
Table 2. Average detected number of bacteria species in children and mothers harboring *F. nucleatum/periodonticum* was significantly higher than that in non-harboring groups.

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<th>Non-harboring</th>
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<tr>
<td>Children</td>
<td>4.8 ± 1.8*</td>
<td>2.7 ± 1.8</td>
</tr>
<tr>
<td>Mothers</td>
<td>5.5 ± 1.8*</td>
<td>3.9 ± 1.9</td>
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*: p<0.001
Table 3. Kappa statistic of detection of targeted 11 periodontal bacteria in 78 children and 68 mothers

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Kappa value</th>
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<tr>
<td>C. sputigena</td>
<td>0.003</td>
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<td>C. gingivalis</td>
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<td>C. ochracea</td>
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</tr>
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<td>C. rectus</td>
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<tr>
<td>F. nucleatum/periodonticum</td>
<td>0.23</td>
</tr>
<tr>
<td>P. nigrescens</td>
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</tr>
<tr>
<td>P. intermedia</td>
<td>0.14</td>
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<td>A. actinomycetemcomitans</td>
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</tr>
<tr>
<td>T. denticola</td>
<td>0.46</td>
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<tr>
<td>T. forsythensis</td>
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<td>P. gingivalis</td>
<td>0.13</td>
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Table 4. Kappa statistic of detection of targeted periodontal bacteria in 10 siblings

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<td><em>C. suptigena</em></td>
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<td><em>C. gingivalis</em></td>
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<tr>
<td><em>C. ochracea</em></td>
<td>0.40</td>
</tr>
<tr>
<td><em>C. rectus</em></td>
<td>0.20</td>
</tr>
<tr>
<td><em>F. nucleatum/periodonticum</em></td>
<td>0.35</td>
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<tr>
<td><em>P. nigrescens</em></td>
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</tr>
<tr>
<td><em>P. intermedia</em></td>
<td>0.88</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>0.35</td>
</tr>
<tr>
<td><em>T. denticola</em></td>
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<tr>
<td><em>T. forsythensis</em></td>
<td>0.11</td>
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<tr>
<td><em>P. gingivalis</em></td>
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Figure Legends

Figure 1. Detection rates of 11 periodontal bacteria in subgingival plaque samples from 78 Japanese children. n: number of examined children.

Figure 2. Detection rates of 11 periodontal bacteria in subgingival plaque samples from 78 children, 68 mothers and child and mother together. n: number of examined children and mothers.
Fig. 1

Prevalence (%)

C. sputigena
C. gingivalis
C. ochracea
C. rectus
F. nucleatum/periodonticum
P. nigrescens
A. actinomycetemcomitans
P. intermedia
T. denticola
T. forsythensis
P. gingivalis

Age (years)

3 (n=10)
5 (n=13)
7 (n=12)
9 (n=8)
Prevalence (%)

- C. sputigena
- C. gingivalis
- C. ochracea
- C. rectus
- F. nucleatum/periodonticum
- P. nigrescens
- P. intermedia
- A. actinomycescomitans
- T. denticola
- T. forsythensis
- P. gingivalis

Child and mother together

Mothers (n=68)
Children (n=78)

Fig. 2