Detection of 6 periodontal bacteria in dental plaque samples from Japanese children

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Abstract We analyzed the distribution of 6 periodontal bacteria (Porphyromonas gingivalis, Prevotella nigrescens, Prevotella intermedia, Eikenella corrodens, Actinobacillus actinomyctetemcomitans and Capnocytophaga sputigena) in dental plaque materials from 227 children (3–6 years old). The plaque materials were collected from all erupted teeth sites using a sterile toothbrush. Chromosomal DNA was extracted from each plaque sample, followed by a polymerase chain reaction with species-specific sets of primers. Standard strains of 6 bacteria were used as controls. Total detection rate of P. gingivalis, P. nigrescens, P. intermedia, E. corrodens, A. actinomyctetemcomitans and C. sputigena were 5.3%, 47.1%, 8.4%, 83.7%, 83.3% and 81.1%, respectively. E. corrodens, C. sputigena and A. actinomyctetemcomitans were very frequently detected at all ages. On the other hand, P. gingivalis and P. intermedia were detected less frequently. Detection rate of P. nigrescens, E. corrodens and C. sputigena increased with age. The average detection number for each age group increased with age (2.63, 2.98, 3.43 and 3.45 for age 3, 4, 5 and 6, respectively). The number of bacterial species in the plaque materials increased with age as well. Our results indicate that P. nigrescens, E. corrodens, A. actinomyctetemcomitans and C. sputigena are established quite early in childhood, these bacteria increase with age in the oral cavity.

Key words Children, Dental plaque, Periodontal bacteria, Polymerase chain reaction (PCR)

Introduction

Dental plaque is the major causative factor of periodontitis1). The major putative pathogens known to be involved in severe periodontitis include Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens (formerly P. intermedia genotype2), Eikenella corrodens, Actinobacillus actinomyctetemcomitans, Capnocytophaga sputigena, Fusobacterium nucleatum, Campylobacter rectus, Tannerella forsythia (formerly Tannerella forsythus)3) and Treponema denticola2,4). Detection of periodontal pathogens in dental plaque is necessary for estimating the risk of periodontal disease development in children.

There have been various methods used for the detection of putative pathogens, including direct microscopy, enzyme tests, enzymelinked immunosorbent assays, DNA fingerprinting and southern blots1,5,6). Recently, several putative periodontal pathogens have been detected in a number of studies using polymerase chain reaction (PCR)1,2,4,7–10). PCR is a powerful tool which can be utilized to address many of research topics. This assay using DNA primers is quick, relatively simple, and able to detect low numbers of bacterial species. 16S ribosomal DNA (rDNA), a small bacterial subunit, can be effectively used for the PCR primers, because it...
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is found universally in all prokaryotic organisms and variable sequence regions are interspersed with highly conserved regions\textsuperscript{7).} It could be suitable for the detection of periodontal pathogens as in the case of children’s dental plaque\textsuperscript{1,2,9,11).} The aim of this study was to examine the detection frequencies of periodontal gram-negative bacteria (\textit{P.gingivalis}, \textit{P.intermedia}, \textit{P.nigrescens}, \textit{A. actinomycetemcomitans}, \textit{E. corrodens} and \textit{C. sputigena}) from dental plaque samples using PCR.

### Materials and Methods

#### Subjects and plaque sampling

Two hundred twenty-seven children aged 3–6 years old (98 males and 129 females) were selected from nursery school children in Nagano Prefecture. All were confirmed as healthy child who doesn’t have general disease and Hellman dental age II A by oral examination mass screening. In performing these operations, the clinician undertook the inspection of the oral cavities in the supine position using dental mirrors according the methods for the survey of dental diseases, checked presence of the plaque adhesion and gingivitis. Collection of dental plaque materials was carried out by the following method: dental plaque materials were recovered in 0.5 ml of gargled liquid saline after 1 minute of tooth brushing by each subject\textsuperscript{12).}

### Genomic analysis

\textit{P.gingivalis} W83, \textit{P.intermedia} ATCC 25611, \textit{P.nigrescens} ATCC 33563, \textit{A. actinomycetemcomitans} JCM8577, \textit{E. corrodens} ATCC 23834 and \textit{C. sputigena} ATCC 33612 were used as controls and grown in Brain Heart Infusion broth (Becton Dickinson Co., MD., USA) supplemented with 0.5% yeast extract, 2.5 mg/ml hemin, 5.0 mg/ml menadione and 0.01% dithiothreitol. All strain were incubated at 37°C anaerobically in a globe box filled with a mixture of gases (N\textsubscript{2}:H\textsubscript{2}:CO\textsubscript{2}, 85:10:5). Chromosomal DNAs were prepared with a Wizard\textsuperscript{®} Genomic DNA Purification kit (Promega Madison, WI, USA) from the incubations and the dental plaque materials. PCR detection was based on the amplification of signature sequences of the bacterial 16S rDNA genes. The primers were shown in Table 1, their specificities and sensitivities for the target organisms having been investigated previously\textsuperscript{13–15).} PCR was performed with the following parameters: a pre-incubation step at 95°C for 5 min, followed by 30 cycles of a denaturing step at 95°C for 30 sec; a primer annealing step at 60°C for 1 min; an extension step at 72°C for 1 min; and 1 cycle of an extra-extension step at 72°C for 2 min. PCR products were examined on 2% agarose gel electrophoresis. The DNA fragments were visualized under a 302 nm ultraviolet light after staining with ethidium bromide.

### Table 1 Oligonucleotides primers used in this study

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Sequence (5’–3’)</th>
<th>Size of amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal primer for positive control</td>
<td>AGAGTTTGATCCTGGCTCAG GCCTACCTTGTTACGACTT</td>
<td>3,480</td>
<td>13</td>
</tr>
<tr>
<td>\textit{P.gingivalis}</td>
<td>TGTAGATGACTGATGTTGAAAAACC ACGTCATCCCCACCTTCC</td>
<td>197</td>
<td>14</td>
</tr>
<tr>
<td>\textit{P.nigrescens}</td>
<td>ATGAAACAAGGTTTTCCGGTAAG CCCAGTCTCTGTGGGCTGGGA</td>
<td>804</td>
<td>15</td>
</tr>
<tr>
<td>\textit{P.intermedia}</td>
<td>TTTGTGGGGAGTAAAGCAGGG TCAACATCTCTGTATCCTGCGT</td>
<td>575</td>
<td>15</td>
</tr>
<tr>
<td>\textit{E.corrodens}</td>
<td>CTATAATCCGATACGGCTTAAAG CTACTAAGCAATCAATTTGCCCC</td>
<td>688</td>
<td>15</td>
</tr>
<tr>
<td>\textit{A. actinomycetemcomitans}</td>
<td>AGAGTTTGATCCTGGCTCAG CACTTAAAGGTCCGCTACGTGCC</td>
<td>593</td>
<td>13</td>
</tr>
<tr>
<td>\textit{C. sputigena}</td>
<td>AGAGTTTGATCCTGGCTCAG GATGCCGCTATATACCATTAGG</td>
<td>185</td>
<td>13</td>
</tr>
</tbody>
</table>
DETECTION OF PERIODONTAL BACTERIA IN DENTAL PLAQUE

Table 2 Percent detection of 6 periodontal bacteria in children by age

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>sub total</th>
</tr>
</thead>
<tbody>
<tr>
<td>(No. subjects)</td>
<td>65</td>
<td>59</td>
<td>72</td>
<td>31</td>
<td>227</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td>3.1</td>
<td>3.4</td>
<td>8.3</td>
<td>6.5</td>
<td>5.3</td>
</tr>
<tr>
<td><em>P. nigrescens</em></td>
<td>35.4</td>
<td>42.4</td>
<td>55.6</td>
<td>61.3</td>
<td>47.1</td>
</tr>
<tr>
<td><em>P. intermedia</em></td>
<td>7.7</td>
<td>5.1</td>
<td>8.3</td>
<td>16.1</td>
<td>8.4</td>
</tr>
<tr>
<td><em>E. corrodens</em></td>
<td>69.2</td>
<td>83.1</td>
<td>93.1</td>
<td>93.5</td>
<td>83.7</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>86.2</td>
<td>79.7</td>
<td>87.5</td>
<td>74.2</td>
<td>83.3</td>
</tr>
<tr>
<td><em>C. sputigena</em></td>
<td>63.1</td>
<td>83.1</td>
<td>90.3</td>
<td>93.5</td>
<td>81.1</td>
</tr>
</tbody>
</table>

There were significant correlations between age and detection of *P. nigrescens* ($P<0.05$), *E. corrodens* ($P<0.05$) and *C. sputigena* ($P<0.05$).

Comparisons were made of detection rate from periodontal bacteria (Table 2), of the frequency of species detection in samples within each age group (Fig. 1) by t-test.

This work was undertaken with the approval of the Ethics Committee of Matsumoto Dental University (No.0013). The consent of the subjects and guardians was obtained after sufficient explanation of the work.

Results

Table 2 shows the percent detection of the 6 periodontal bacteria in plaque materials taken from 227 subjects by age. PCR analysis using 16S rDNA primers confirmed the presence of bacteria in all plaque materials. There were significant correlations between age and detection of *P. nigrescens* ($P<0.05$), *E. corrodens* ($P<0.05$) and *C. sputigena* ($P<0.05$). *E. corrodens*, *C. sputigena* and *A. actinomycetemcomitans* were found in approximately 80–90% in total age. *E. corrodens* and *C. sputigena* increased with age (in the range of 69.2% and 63.1% in 3 years old to both 93.5% in 6 years old, respectively). In contrast, detection rate of *A. actinomycetemcomitans* was settled in all age 86.2, 79.7, 87.5 and 74.2% for age 3, 4, 5 and 6, respectively. An obligate anaerobe, *P. gingivalis* and *P. intermedia* were detected relatively infrequently at all ages, with the percent range of positive subjects from 3.1 to 6.5% and 7.7 to 16.1%, respectively. On the other hand, it was noted that the detection rate of *P. nigrescens* positive subjects increased with age, and reached about 60% in the materials from those 6 years old 35.4, 42.4, 55.6 and 61.3% for age 3, 4, 5 and 6, respectively. *P. intermedia* and *P. nigrescens* belong to Prevotella species, however total detection rate of these bacteria were significant ($P<0.01$).

The kind number of bacterial species detected in individual plaque materials was investigated for each age group (Fig. 1). Two children in 3 age (3.1%) and 3 children in 4 age (5.1%) showed none of the target bacteria in their plaque materials (Fig. 1a and b). Anyone was not detected all kinds of 6 bacteria. In subjects with the 6 bacteria, the average number in each age group was 2.63, 2.98, 3.43 and 3.45 for age 3, 4, 5 and 6, respectively (Fig. 1a–d). The values increased with age. A significant difference in the average number was found between the aged 3 and 5 years ($P<0.01$), between the aged 3 and 6 years ($P<0.01$), between the aged 4 and 5 years ($P<0.01$) and between the aged 4 and 6 years ($P<0.01$). The results showed there was a relation between the detected number of bacterial species and age.

Discussion

As shown in Table 2, the detection rate of *P. nigrescens* *E. corrodens* and *C. sputigena* increased with age. On the other hand, detection of *A. actinomycetemcomitans* did not change with age. This bacteria was detected in many subjects from an early age, and the total detection rate was 83.3%. Kimura et al. and Ooshima et al. reported detection of this bacteria aged 3 to 6 was 40 to 60%2,4. Periodontal bacteria, such as *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans*, were carried by family members10, thus the detection rate in this study...
was high average. And *A. actinomycetemcomitans* was detected from saliva, dental plaque, tooth, and tongue at an early age\(^4,16\). These results suggest this bacteria may be an early acquisition in the oral cavities of children. *E. corrodens* and *C. sputigena* were very frequently detected at all ages. Ooshima *et al.* examined these bacteria frequently found in dental plaque and saliva\(^4\). These results suggest *E. corrodens* and *C. sputigena* may be part of the oral flora. *A. actinomycetemcomitans, E. corrodens* and *C. sputigena*, a facultative anaerobe, were found in about 60% of the plaque samples from all age groups in our study, suggesting that the colonization of some putative periodontal bacteria could occur quite early in childhood without clinical signs of periodontal disease.

*P. intermedia* and *P. nigrescens* (the former *P. intermedia* genotype 2) low a 94% similarity and 6.3% difference in 16S rRNA genes\(^2,17\). These organisms are frequent inhabitants of periodontal pockets and are difficult to distinguish by conventional culture identification\(^18\). *P. nigrescens* and *P. intermedia* were detected in 47.1% and 8.4%, respectively (\(P<0.01\)). In another study, the results for these bacteria were different. In recent studies, *P. nigrescens* in plaque increased with age, reaching 73% at 12 years of age\(^4\), or in another study 100%\(^1\). There was an increased detection rate of *P. nigrescens* in plaque, and the organism may be a common member of the oral microbial flora at a very early age. *P. intermedia* was rarely detected in total materials. Ashimoto *et al.* reported a prevalence of *P. intermedia* in pediatric gingivitis subjects aged 2 to 11 was 18%\(^15\). Okada *et al.* suggested a figure of 6.7% for 2 to 12 year olds.
with dental plaque\textsuperscript{11}, while Ooshima et al. put the figure at 10% at 2 to 15 year olds\textsuperscript{4}. \(P\).intermedia is typically not found in the primary dentition of most children. \(P\).gingivalis was also rarely detected in total materials. The rate increased slightly with age, and reached 6.5% in 6 year olds. This organism was not detected in periodontally healthy children\textsuperscript{9}, and in only 4.8% using a PCR method\textsuperscript{1}. Furthermore, Conrads et al. reported this bacteria was not found in children from 3 to 10 years of age\textsuperscript{13}. \(P\).gingivalis has been found almost exclusively in active sites of deep pockets of periodontally diseased patients, while \(P\).intermedia has been cultured in both healthy and diseased sites, with \(P\).nigrescens predominating in healthy sites in adults\textsuperscript{18}. This is similar to the distribution of these bacteria in children\textsuperscript{11}.

We examined the number of bacterial species detected in each plaque materials by age (Fig. 1). These results indicate 5 out of 227 children (2.2%) did show any kind of the periodontopathic bacteria. The average number in each age group increases gradually by age (\(P<0.05\)). Kimura et al. suggested the number of bacterial species in the plaque samples increased with age until 5 years old, and then reached a plateau after the mixed dentition period\textsuperscript{2}.

Toothbrushing has been widely accepted as a method for removal of dental plaque. It is easy to get dental plaque for the examination of periodontal...
pathogens. The consent of parents can be more easily obtained than for using a curette, paper point or excavators. Our results show brushing detected more facultative anaerobes than obligate anaerobes. When the materials were collected by toothbrush, supragingival plaque predominated over subgingival plaque. In the future, we would like to examine other periodontal bacteria, such as C. rectus, T. denticola, F. nucleatum and T. forsythia, and investigate periodontal organisms for wide age ranges.

Conclusion

We examined the detection of 6 periodontal bacteria in dental plaque using PCR. A. actinomycetemcomitans was high detected in all age. E. corrodens and C. sputigena were increased with age, reaching more than 90% at 6 years old. P. gingivalis and P. intermedia were detected less frequently. P. nigrivores was increased with age, reaching more than 60% at 6 years old. These results suggest E. corrodens and C. sputigena A. actinomycetemcomitans and P. nigrivores could be early colonizers of periodontopathic bacteria. The average detection number for each age group increased with age (2.6, 2.4, 3.3 and 3.5 for age 3, 4, 5 and 6, respectively). The number of bacterial species in the plaque materials increased with age.

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