# The Detection of *Porphyromonas gingivalis, Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in tooth, tongue and buccal mucosa plaques in children, using immunoslot blot assay(IBA)

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*The present study was to investigate the distribution of typical periodontpathic bacteria (Porphyromonas gingivalis, Prevotella intermedia and Actinobacillus actinomycetemcomitans) in tooth, tongue and buccal mucosa plaques in 3 to 17 Year old children. Clinical parameters (Rates of df, d, DMF, and D; plaque and gingival index) for each subject were determined prior to the collection of each site plaque. Three periodontopathic bacteria on each site samples were detected using IBA. The frequency of three bacteria for tooth plaque was higher than that for tongue or buccal mucosa plaque. The frequency of Porphyromonas gingivalis and Prevotella intermedia in supragingival plaques was significantly higher than that of corresponding ones in tongue or buccal mucosa plaques. The three bacteria also occurred more frequently in subjects aged between 10 and 14 years. Periodontopathic bacteria may be enhanced in circumpubertal children.*
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## INTRODUCTION

An earlier study by Tanaka investigated the frequency of *Porphyromonas gingivalis* on tongue, buccal mucosa, supra and subgingival dental plaques and occlusal surfaces of teeth in adult periodontal patients using an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody. Results indicated that *Porphyromonas gingivalis* was detected on each site of the oral cavity with the highest frequency detected in subgingival plaque.[1] Also we previously reported a relationship between the frequency of reactivity 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 plaques in 6 to 9 year-old children harbor *Porphyromonas gingivalis*.[2] In addition, we recently reported that supragingival plaques in children with and without active caries can harbor periodontopathic bacteria such as *Porphyromonas gingivalis, Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*.[3] However, there are few studies about the distribution of periodontopathic bacteria in the oral cavity at different ages.[4-5] The high incidence of periodontopathic bacteria in children may become a risk factor of periodontal disease in future. Thus, the purpose of the present study

was to investigate the distribution of the oral cavity in children of 3 periodontopathic bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in tooth, tongue and buccal mucosa plaques in children aged 3 to 17 years using an immunoslot blot assay system. This system has been useful in practice because it is a convenient but precise procedure for rapid detection of periodontopathic bacteria.

## MATERIALS AND METHODS

Sixty children (37 boys and 23 girls aged between 3.0 and 17.0 years ) who presented at the Meikai University Hospital in Saitama prefecture, Japan were used. Each patient was charted on dmf's (in df: d-decayed primary teeth indicated for filling; f-filled primary teeth and DMF: D-Decayed teeth; M-Missing teeth; F-Filled teeth in the permanent dentition), plaque index[6] and gingival index[7] prior to the collection of plaque samples from the tooth surface (supragingival plaques), tongue and buccal mucosa plaques.

Exclusion criteria included antibiotic therapy in the previous 3 months, any systemic conditions which could influence the course of oral diseases or which would require pre-medication for monitoring procedures. Informed consent was obtained from children and each children's parents.

Ethical clearance for the study was obtained from the local ethics committee.

### Sample collection

Tooth samples (supragingival plaques) from each subjects were obtained from the mesiobuccal surfaces of second primary molar and first permanent molars. Tongue and buccal mucosa samples was obtained by a loopful (Catalog no. 254410; Nunk Co., Copenhagen, Denmark) scraped over the tongue dorsum and the same buccal mucosa adjacent to the teeth selected for supragingival plaque samples. Thereafter, each loopful of the collected plaques was transferred to microtubes containing 500μl of sodium carbonate buffer (pH 9.6).

The collected plaque samples from each subject were pooled at –80ºC and then were thawed just before being analyzed separately by immunoslot blot assay. The plaque samples were dispersed by pulsed sonication (Sonifier cell disrupter; Branson Sonic Power Co.,Danbury, Conn.) for 10 seconds at 40 W. The dispersed samples (25μl) were used for the immunoslot blot assay.[8]

### Bacterial strains

*P gingivalis* 381, *P intermedia* ATCC 25611 and *A actinomycetemcomitans* Y4 were used in this study.

### Monoclonal antibodies

The following monoclonal antibodies specific for their respective bacteria were selected for use in this study[8,9]? monoclonal antibody BGF7 for *P gingivalis*, BIF6 antibody for *P intermedia* and AAY4 antibody for *A actinomycetemcomitans*.

### Immunoslot blot assay

This was performed using the procedure previously described.[2,3,8] Briefly, supragingival plaque samples (25μl) suspended in 500μl sodium carbonate buffer or each sonicated bacterial extract (25ng of protein) was blotted onto nitrocellulose paper (Bio-RaD, Tokyo, Japan) in each well of an immunoslot blot apparatus (Hybri-slot 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 (Tokyo Kasei Kogyo Co., Tokyo, Japan; PBS-Tween). Afterwords, 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 next incubated for 60 min with a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Bio-Rad), washed and then visualized 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 $1x10^4$ cells of each periodontopathic bacterium, according to the author's previous investigation[8] related to the degree of colonization and the 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 by the General Linear Models procedure and ANOVA for multiple comparisons.

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

## RESULTS

The mean±standard deviation value of each clinical parameter for age group related subjects is shown in Table 1. As a result, the rates of df declined respectively in the 5-9 age group, followed by the 10-14 age group and the 0-4 age group. Also, the rates of DMF declined respectively in the 15-19 age group followed by the10-14 age group and the 5-9 age group. The only rate of df between 5-9 age group and 10-14 age group were statiscally significant (Scheffe's analysis, $p<0.05$) and there were no siginificant differences between the groups in the other clinical parameters.

**Table 1 Clinical parameters,i.e., df, d, DMF, D as a function of age groups**

| | |
|---|---|
| 0～4 (age group) | |
| Rates of df | 28.00±30.12 |
| Rates of d | 7.00±8.37 |
| 5～9 (age group) | |
| Rates of df | 60.24±30.15 * |
| Rates of DMF | 7.60±16.50 |
| Rates of d,D | 5.40±9.50 |
| 10～14 (age group) | |
| Rates of df | 31.30±44.93 |
| Rates of DMF | 15.81±20.34 |
| Rates of d,D | 6.09±9.66 |
| 15～19 (age group) | |
| Rates of DMF | 25.71±17.20 |
| Rates of D | 11.43±10.23 |

mean ±standard division
ANOVA Scheff analysis,p<0.05*

The relationship between the number of 3 periodontopathic bacteria positive subjects and the degree of plaque index (PI) in each site is shown in Figure 1. Positive subjects were observed in all sites in PI-1 and PI-2 groups. However, no positive subjects in tongue and buccal mucosa was observed in the PI-3 group subjects. Positive subjects for *Porphyromonas gingivalis*, *Prevotella intermedia* or *Actinobacillus actinomycetemcomitans* were seen more often in tooth plaques (supragingival plaques) than those for corresponding bacteria in tongue or buccal mucosa plaques. We examined also the relationship between the number of positive subjects reactive to 3 periodontopathic bacteria

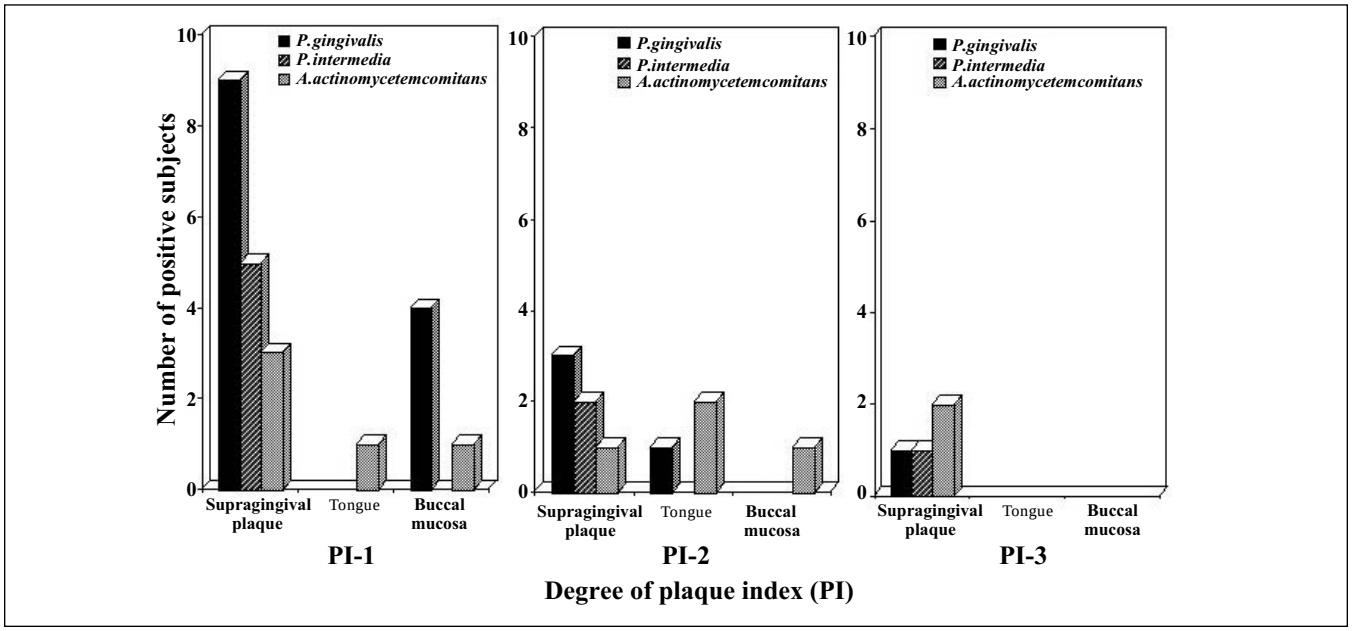


**Figure 1.** Number of periodontopathic bacteria positive subjects (Pg, Pi or Aa) for PI-1, PI-2 and PI-3 as a function of supragingival plaque, tongue or buccal mucosa

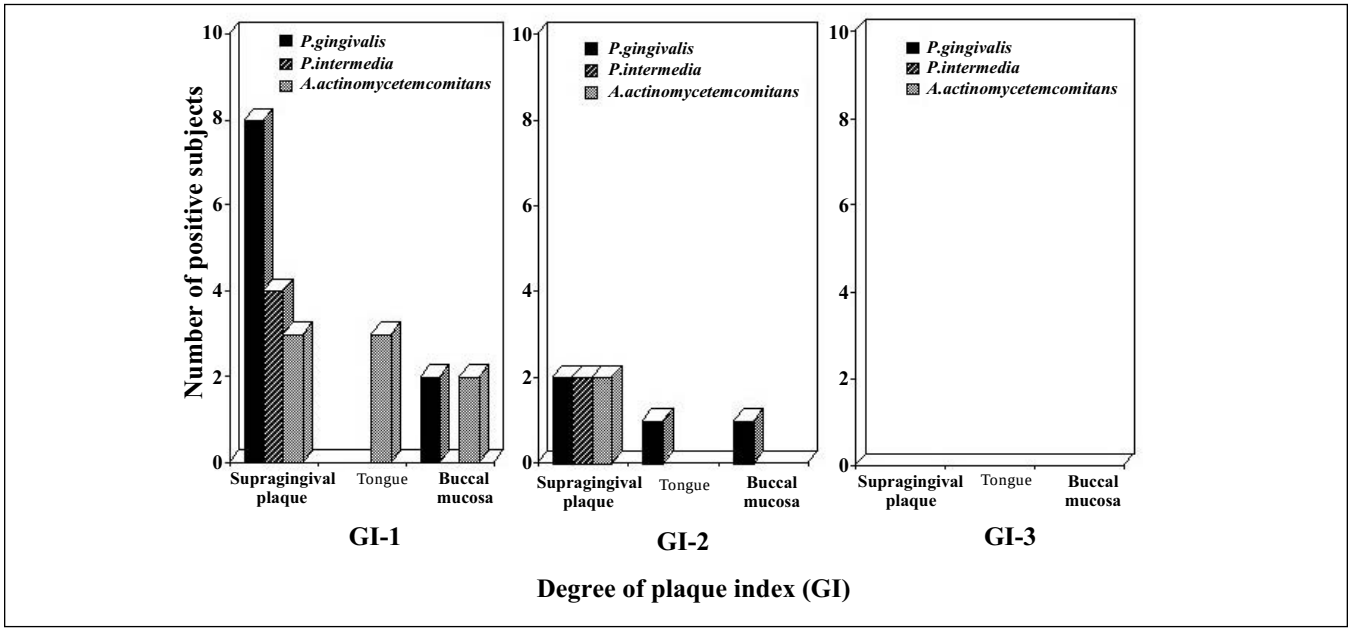


**Figure 2.** Number of periodontopathic bacteria positive subjects (Pg, Pi or Aa) for GI-1,GI-2 and GI-3 as a function of supragingival plaque, tongue or buccal mucosa

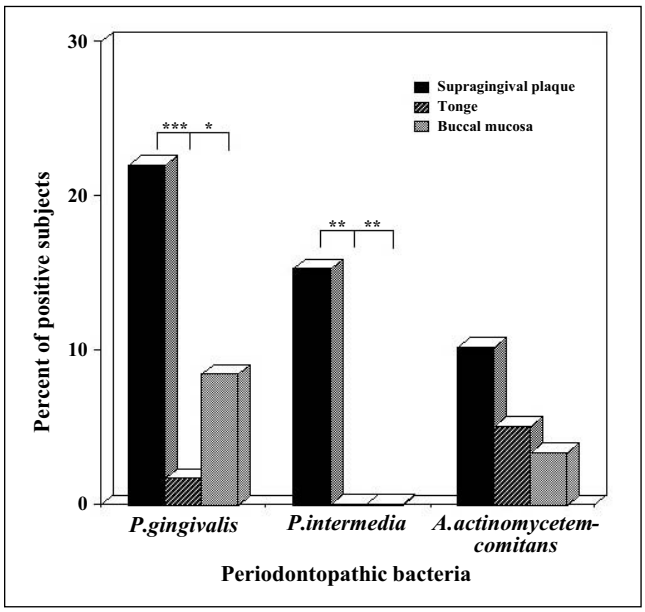


**Figure 3.** Percent of positive subjects for supragingival plaque, tongue or buccal mucosa as a function of P. gingivalis, P. intermedia and A. action-mycetemcomitans P gingivalis: supragingival plaque vs. tongue or buccal mucosa P intermedia: supragingival plaque vs. tongue or buccal mucosa $\chi^2$-test, p<0.05*, p<0.01**, p<0.001***

and the degree of gingival index (GI) in each sites (Figure 2). The positive subjects were obseved in both the GI-1 and GI-2 group subjects but were not observed in GI-3 group subjects. The positive subjects for *Porphyromonas gingivalis*, *Prevotella intermedia* or *Actinobacillus actinomycetemcomitans* in each sites plaques were also highly observed in teeth plaques (supragingival plaques), but were not found in tongue and buccal mucosa plaques. Positive subjects were not found in the GI-3 group subjects. The relationship between the percentage of bacteria-positive subjects and three periodontopathic bacteria in each site plaques is shown Figure 3.

The percentage of positive subjects for *Porphyromonas gingivalis* and *Prevotella intermedia* in supragingival plaques was significantly higher than that for corresponding ones in tongue or buccal mucosa plaques. A significant difference between the supragingival plaques and the tongue or the buccal mucosa plaques was found ($\chi^2$-test, p<0.05,p<0.01, p<0.001). However, such a relationship was not found in *Actinobacillus actinomycetemcomitans*.

We also examined the distribution of the three species by age in this study. The relationship between the three periodontopathic bacteria positive subjects and the age groups in each subjects is shown in Figure 4.

As a result, the positive subjects for 3 periodontopathic bacteria was the highest in 10-14 age groups, followed by 5-9 age groups.

The minimum age of subjects positive for *Porphyromonas gingivalis*, *Prevotella intermedia* or *Actinobacillus actinomycetemcomitans* was 6 years and 3 months, while the maximum age was 15 years and 5 months.

## DISCUSSION

The author previously reported that the frequency of *P gingivalis* in plaques from five sites (i.e: supra-and subgingival plaque, tongue, buccal mucosa, and occlusal surface) in the oral cavity of adult periodontal patients by using an enzyme linked immunosorbent assay (ELISA) with *P gingivalis*–specific monoclonal antibody showing the highest reactivity for *P gingivalis* was detected in subgingival plaques.[1] This finding was

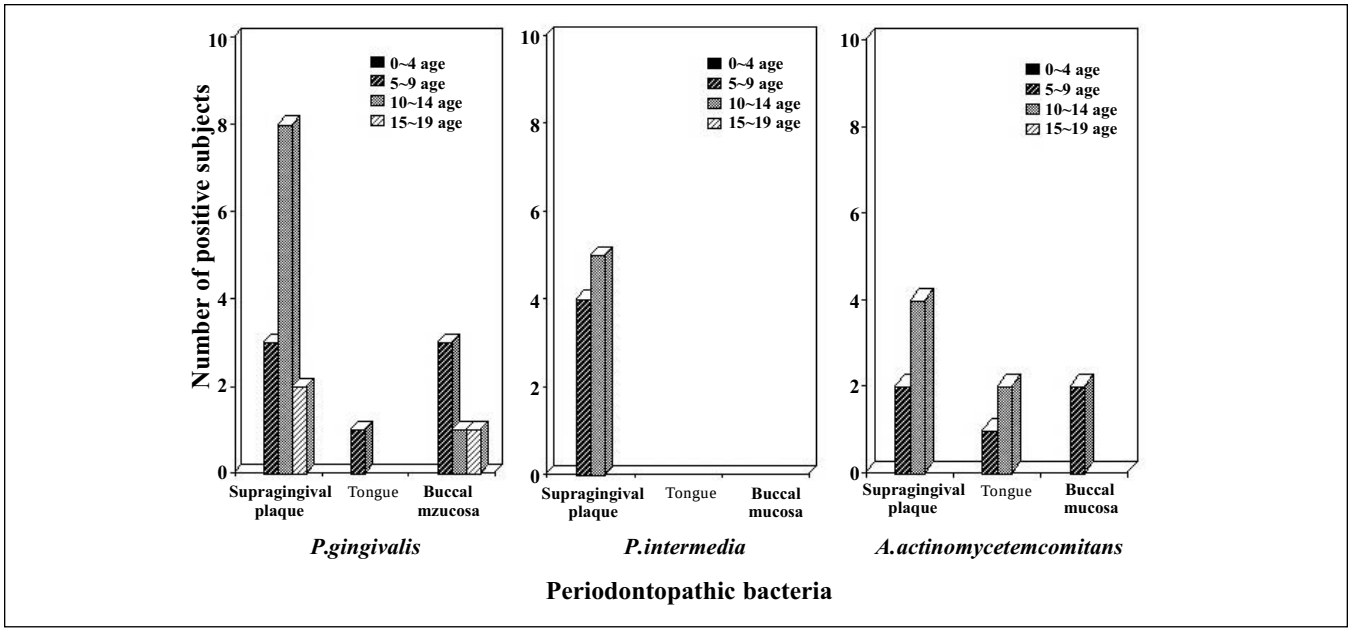


**Figure 4.** Number of periodontopathic bacteria positive subjects in age group as a function of supragingival plaque, tongue or buccal mucosa

consistent with the results of Zambon *et al*. who examined the intraoral distribution of *P gingival* is in black pigmented *Bacteroides* spp. in the human oral cavity using anaerobic technique.[2] These findings suggest that periodontopathic bacteria such as *P gingivalis* harbor the distribution in plaque samples of oral cavity.

Also, we recently reported a relationship between the frequency of reactivity for *P gingivalis* and *Prevotella* species in supra and subgingival plaques and periodontal clinical parameters according to the subject's age. This finding suggested that the supragingival plaques in 6-to 9-year-old children harbor *P gingivalis* and additionally we found that *P gingivalis*, *P intermedia* and *A actinomycetemcomitans* were detected in the supragingival plaques of children at an early age and that *P gingivalis* was detectable at a broader age range.[3] This finding suggested the necessity to continue to monitor positive individual for these periodontopathic bacteria, since their risk of developing periodontal diseases may be increased in the future. A longitudinal investigation would be particularly important to monitor these bacteria during the transition from periodontal health to disease.

Tanner *et al* recently reported that the frequency of 38 microbial species was detected in 171 randomly selected children from 6 to 36 months of age and oral samples were assayed by means of a checkerboard DNA probe assay.[4]

The detection frequencies from tongue samples in children under 18 months were *P gingivalis* by 23%, *A actinomycetemcomitans* by 30%,with similar detection frequencies in children over 18 months. Thus periodontal pathogens such as *P gingivalis* were found to be detected even in the youngest subjects. Most species that were detected more frequently were from tongue than from tooth samples in children under 18 months suggesting that the tongue is a potential microbial reservoir recovering *P gingivalis*, *A actinomycetemcomitans*, *P intermedia*.[10-12]

Also, Frisken *et al.*[13] previously demonstrated that anaerobic bacteria can successfully colonize young children before tooth eruption. The higher species-detection frequencies from tongue compared with that in tooth-associated samples have been reported for *A actinomycetemcomitans*, *P gingivalis*, and *P intermedia* by means of immunofluorescence,[14] for *P intermedia* and *P nigrescens* by means of AP-PCR,[15] and for *A actinomycetemcomitans* by means of selective culture.[17] These findings suggest that the dorsum of the tongue houses organized

Van der Velden *et al.*[17] previously showed that the presence of motile organisms and black pigmented *Bacteroides* on the mucosal surfaces of the tongue and tonsils was correlated with the presence of these microorganisms in the 23 day old dental plaque. Together with these finding, it was concluded that, in particular, the mucosa of tongue and tonsils may harbor periodontopathic microorganisums and may possibly function as a nest for these bacteria. McClellan *et al.*[18] also previously analyzed samples harvested using paper points from the gingival sulcus of every tooth, from the buccal mucosa and from fissures of the dorsum of the tongue from 198 individuals between the ages of 20 days and 18 years using a PCR-based method that is capable of detecting as few as 10 *P gingivalis* cells. *P gingivalis* was reported to be found in samples from all ages including one infant 20 days old and three of six other pre eruptive infants. The overall colonization rate for this population was 37% compared to a colonization rate of 46% in 380 adults over 20 years of age determined using the same methodology. No association between age and colonization was found. These observations suggest that colonization occurs early in life and is maintained at similar frequencies throughout infancy, childhood and adolescence. Yang *et al.*[5] recently reported that 71% (66/93) of the 18- to 48-month-old children were infected with at least one periodontal pathogen. In addition, *P gingivalis were* detected in 68.8% ( 64/93).

Also, we recently reported that *P gingivalis*, *P intermedia*, and *A actinomycetemcomitans* were detected in the supragingival plaque of children with and without caries and the three periodontopathic bacteria such as *P gingivalis*, *P intermedia*, and *A actionmycetemcomitans* were found at an early age (3.6 years). [3] These findings are consistent with those of Tanner et al.[4]

The main reservoir of periodontopathic bacteria is in the human oral cavity, i.e., supra- and subgingival, the tongue, buccal mucosa and the tonsillar plaques, resulting from the evidence that *A actinomycetemcomitans* can be transmitted from parent to child and also *P gingivalis* can be transmitted among individuals in a household.[19]

In conclusion, the distribution of three periodontopathic bacteria in the oral cavity in children was observed in plaques for tongue and buccal mucosa, suggesting that also tongue and buccal mucosa may be a potential microbial reservoir for tooth-associated species. Periodontopathic bacteria may be much more enhanced in circumpubertal children.

## CONCLUSIONS

There was a significant difference in the rates of df between 5-9 age group and 10-14 age group (Scheffe's analysis, $p<0.05$ ). Positive subjects were observed in all of each sites of the PI-1 and PI-2 group subjects. However, any positive subjects in tongue and buccal mucosa was not observed in the PI-3 group subjects. Similarily, the positive subjects were obseved in both the GI-1 and GI-2 group subjects but were not observed in GI-3 group subjects.

The percentage of positive subjects for *P gingivalis* and *P intermedia* in supragingival plaques was significantly higher than that in tongue or buccal mucosa

plaques. A significant difference between supragingival plaques and tongue or buccal mucosa plaques was found.

The positive subjects for three periodontopathic bacteria was the highest in 10-14 age groups. Periodontopatic bacteria may occur more frequently in circumpubertal children. Periodontopathic bacteria was observed in tongue and buccal mucosa plaques as well as in tooth. Tongue and buccal mucosa was a potential microbial reservoir for tooth-associated species.

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**REFERENCES**

1. Tanaka S. Application of Bacteroides gingivalis- specifc monoclonal antibody for immunodiagnosis of adult periodontal disease. Bull Josai Dent Univ 14: 444–457, 1985.
2. Tanaka S, Murakami Y, Ogiwara T, et al. The prevalence of Porphyromonas gingivalis and Prevotella spp. in supra- and subgingival plaques, and periodontal clinical parameters according to subject age. J Periodontol 73: 877–885, 2002.
3. Tanaka S, Yukio M, Kazuhito S, et al. The detection of Porphyromonas gingivalis, Prevotella intermedia, and Actinobacillus actinomycetem-comitans in the supragingival plaque of children with and without caries. Pediatric Dent. 25:143–148, 2003.
4. Tanner ACR, Milgrom PM, KentJr R, et al. The microbiota of young children from tooth and tongue samples. J Dent Res. 81: 53–57, 2002.
5. Yang EY, Tanner ACR, Milgrom P, et al. Periodontal pathogen detection in gingival/tooth and tongue flora samples from 18-to 48-month-old children and periodontal status of their mothers. Oral Microbiol Immunol 17:55–59, 2002.
6. Silness J, Loe H. Periodontal disease in pregnancy: Correlation between oral hygiene and periodontal condition. Acta Odontol Scand 22: 121–135, 1964.
7. Loe H, Silness J. Periodontal disease in pregnancy: Prevalence and severity. Acta Odontol Scand 21: 533–551, 1963.
8. Hanazawa S, Tanaka S, Kin M, et al. Application of monoclonal antibodies to the detection of black- pigmented Bacteroides spp. in subgingival plaques by immunoslot blot assay. J Clin Microbiol 28: 2248–2252, 1990.
9. Hanazawa S, Saitoh K, Ohmori Y, Nishihara H, Fujiwara S. and Kitano S. Production of monoclonal antibodies that recognize specific and cross-reactive antigen of Bacteroides gingivalis. Infect Immun 46: 285–287, 1984.
10. Lee KH, Tamer ACR, Maiden MFJ and Weber HP. Pre- and post-implantation microbiota of the tongue, teeth, and newly-placed implants. J Clin Periodontol 26: 822–832, 1999.
11. Asikainen S, Alaluusua S and Saxen L. Recovery of Actinobacillus Actinomycetemcomitans from teeth, tongue and saliva. J Periodontol 62: 203–206, 1991.
12. Van Winkelhoff AJ, Van derVelden U, Winkel EG and de Graaf J. Black- pigmented Bacteroides and motile organisms on oral mucosal surfaces in individuals with and without periodontal break-down. J Periodont Res 21:434–439, 1986.
13. Frisken KW, Higgins T, Palmer JM. The incidence of periodontopathic microorganismus in children. Oral Microbiol Immunol 5: 43–45, 1990.
14. Timmerman MF, van der Weijden G, Armand S, et al. Untreated perio-dontal disease in Indonesian adolescents. Clinical and microbiological baseline data. J Clin Periodontol 25:215–224, 1998.
15. Matto J, Saarela M, Troil-Linden B,et al. Similarity of salivary and subgingival Prevotella intermedia and Prevotella nigrescens isolates by arbitrarily primed polymerase chain reaction. Oral Microbiol lmmunol 11:395–401, 1996.
16. Muller HP, Zoller L, Hoffmann S, Lobinsky D. Natural distribution of Actinobacillus Actinomycetemcomitans in young men with minimal periodontal disease. J Periodont Res 31:373–380, 1996.
17. Van der Velden U, Van Winkelhoff AJ, Abbas F and de Graaf J. The habitat of periodontopathic micro-organisms. J Clin Periodontol 13: 243–248, 1986.
18. McClellan DL, Griffen AL, Leys EJ. Age and prevalence of Porphyromonas gingivalis in children. J Clin Microbiol 34: 2017–201, 1996.
19. Genco RJ. Consensus report on periodontal diseases: Pathogenesis and microbial facters. Ann Periodontol.1: 926–932, 1996.