Journal of Oral Science, Vol. 54, No. 1, 77-83, 2012

Original

Prevalence of *Porphyromonas gingivalis fimA* genotypes in Japanese children

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(Received 21 September 2011 and accepted 14 February 2012)

Abstract: ***Porphyromonas gingivalis*** **FimA fimbriae have been classified into 6 genotypes (types I–V and Ib) based on the diversity of the *fimA* genes encoding the fimbrial subunits. We investigated the prevalence of *fimA* genotype in Japanese children. Dental plaque specimens were obtained from 400 subjects (age; 2 to 15 years), including 134 with healthy gingiva, 239 with gingivitis and 27 with periodontitis, and then analyzed by polymerase chain reaction. *P. gingivalis* was detected in 1.5%, 10.0% and 29.6% of these subjects, respectively. Significant differences were observed with regard to *P. gingivalis* infection among the groups [chi-squared analysis: gingivitis vs. healthy, $P < 0.01$, odds ratio (OR) = 7.4; periodontitis vs. healthy, $P < 0.001$, OR = 27.8]. In *P. gingivalis*-positive subjects with periodontitis, the most prevalent *fimA* types were type Ib/type II combination (37.5%) and type IV (37.5%), followed by type II (25.0%), while type IV (33.3%) and type II (29.2%) were most often detected in those with gingivitis. Our results suggest that the presence of *P. gingivalis* is associated with periodontal diseases, and that the type II, IV and Ib/II combination are the most common among *fimA* genotypes. (J Oral Sci 54, 77-83, 2012)**

Keywords: *Porphyromonas gingivalis*; children; *fimA*; genotype; periodontal condition.

Introduction

Periodontal diseases are mainly associated with gram-negative bacteria that initiate a series of events leading to the loss of periodontal attachment and alveolar bone surrounding teeth (1). Among these pathogens, *Porphyromonas gingivalis* is considered to be one of the most important oral cavity infectious agents, as it can cause several types of periodontal diseases (2). *P. gingivalis* is uncommon or found in low numbers in healthy individuals and those with gingivitis, while it is more frequently detected in those with more destructive forms of disease (3). The pathogen is considered to be mainly associated with adult periodontitis (4), and we previously found that *P. gingivalis* organisms were rarely present in the oral cavities of healthy children (5).

P. gingivalis has been shown to produce a number of virulence factors, including fimbriae, lipopolysaccharide, capsules and proteases (6). In particular, FimA fimbriae, filamentous components on the cell surface, are thought to play important roles in the colonization and invasion of periodontal tissue (7,8). The *P. gingivalis fimA* gene has been classified into six variants (types I through V and Ib) based their nucleotide sequences (9-11). A polymerase chain reaction (PCR) method with *fimA* type-specific primer sets has been developed to differentiate the six types of *fimA* genes of *P. gingivalis* in saliva and dental plaque samples collected from periodontitis patients (11,12). Furthermore, the infectious traits of this bacterium that have been reported to have an influence

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Table 1 *fimA* type-specific and 16S ribosomal RNA-specific primers used in this study

Primer pairs	Direction	Sequence	Size (bp)[a]	Reference
P. gingivalis 16S ribosomal RNA	forward	AGG CAG CTT GCC ATA CTG CG	404	19
	reverse	ACT GTT AGC AAC TAC CGA TGT		
Type I *fimA*	forward	CTG TGT GTT TAT GGC AAA CTT C	392	12
	reverse	AAC CCC GCT CCC TGT ATT CCG A		
Type Ib *fimA*	forward	CAG CAG AGC CAA AAA CAA TCG	271	11
	reverse	TGT CAG ATA ATT AGC GTC TGC		
Type II *fimA*	forward	ACA ACT ATA CTT ATG ACA ATG G	257	12
	reverse	AAC CCC GCT CCC TGT ATT CCG A		
Type III *fimA*	forward	ATT ACA CCT ACA CAG GTG AGG C	247	12
	reverse	AAC CCC GCT CCC TGT ATT CCG A		
Type IV *fimA*	forward	CTA TTC AGG TGC TAT TAC CCA A	251	12
	reverse	AAC CCC GCT CCC TGT ATT CCG A		
Type V *fimA*	forward	AAC AAC AGT CTC CTT GAC AGT G	462	10
	reverse	TAT TGG GGG TCG AAC GTT ACT GTC		
Eubacterial 16S ribosomal RNA	forward	CAG GAT TAG ATA CCC TGG TAG TCC ACG C	625	12
	reverse	GAC GGG CGG TGT GTA CAA GGC CCG GGA ACG		

a: Expected size of PCR product

on periodontal health status can be differentiated based on clonal variations of the *fimA* genes (13).

The prevalence of *fimA* genotypes in adults is reportedly related to periodontal condition. However, few investigations have noted a relationship between the prevalence of *fimA* genotypes and periodontal health status in children (14). The purpose of the present study was to investigate the prevalence of *fimA* genotype in Japanese children.

Materials and Methods

Subjects

Four hundred children, aged 2 to 15 years, who were patients at the Pediatric Dental Clinic of Hiroshima University Hospital, Japan, were enrolled in the present study. Consent for participation was obtained from at least one of their parents prior to the study according to the ethical guidelines of the Declaration of Helsinki (1975) and ethical clearance was obtained from the Ethical Committee of Hiroshima University (Epidemiology-No. 52/2). Subjects with clinically healthy gingiva, as well as those with gingivitis and periodontitis, were studied and classified as follows (5). Those with an absence of inflammation, no bleeding on brushing, and no radiographic bone loss were regarded as having clinically healthy gingiva and classified as the healthy group (n = 134), while subjects with mild to moderate inflammation at more than 1 site, as determined by gingival index (15), and no radiographic bone loss were classified as the gingivitis group (n = 239), and those with attachment loss of greater than 3 mm at 1 site or more among four teeth were defined as the periodontitis group (n = 27). Children with systematic diseases were not excluded, although subjects who had taken antibiotics within 3 months prior to the study were excluded.

Plaque sampling and genomic DNA preparation

Dental plaque was collected from all erupted teeth by brushing with a sterile toothbrush for 1 minute, in accordance with a previously described method (5). Plaque samples were immediately transported to the laboratory and stored at -20°C before extraction of genomic DNA. Genomic DNA from each plaque sample was obtained using a standard miniprep procedure, as reported previously (16), to which we added RNase treatment (17).

PCR

Table 1 shows the PCR primers used in the present study. A PCR method was used to detect *P. gingivalis* (16S ribosomal RNA), and *fimA* typing was then performed using species-specific primers, as described previously (10-12,18). All primers were purchased from Sigma-Aldrich Japan (Tokyo, Japan). We also detected eubacterial 16S ribosomal RNA (GenBank accession number M75035) using a method described previously by Goncharoff et al. (19). Eubacterial 16S ribosomal

Table 2 Distribution of *P. gingivalis* in children divided into healthy, gingivitis, and periodontitis groups, and probability of *P. gingivalis* detection

	P. gingivalis				95% C. I. for odds ratio	
	Undetected	Detected (%)	Chi-square	Odds ratio	Lower	Upper
Healthy	132	2 (1.5)				
Gingivitis ‡	215	24 (10.0)	9.68 **	7.4	1.7	31.7
Periodontitis ‡	19	8 (29.6)	30.54***	27.8	5.5	140.8

‡ vs. Healthy group, **: $P < 0.01$, ***: $P < 0.001$

Table 3 Distribution of *P. gingivalis* in children with healthy, gingivitis, and periodontitis in different dentition stages

	Number positive subjects/sampled subjects (%)		
Dentition (number of subjects)	Healthy	Gingivitis	Periodontitis
Primary (125)	0/74 (0.0)	2/44 (4.5)	1/7 (14.3)
Mixed (215)	2/47 (4.3)	17/152 (11.8)	4/16 (25.0)
Permanent (60)	0/13 (0.0)	5/43 (11.6)	3/4 (75.0)
Total (400)	2/134 (1.5)	24/239 (10.0)	8/27 (29.6)

RNA primers that match nearly all bacterial 16S rRNA genes were used (18). PCR amplification was performed in a reaction mixture (25 µL) consisting of PCR beads (GE Healthcare UK Limited, Buckinghamshire, UK) containing enzyme (two units of *Taq* DNA polymerase) and the required reagents, as well as 25 pmol of each primer, and 20 to 50 ng of template DNA solution in a thermal cycler (DNA Engine PTC-220 DYAD; MJ Research, Waltham, MA, USA). Each set of PCR analyses included a negative control (water blank) in addition to the positive control. Reaction mixtures were denatured at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with a final cycle of 72°C for 7 min (13). After amplification, PCR products for *fimA* type Ib were digested with *Rsa*I (11). Next, 15 µL of each PCR product was analyzed by electrophoresis on a 1.2-2.0% agarose gel and the newly synthesized DNA fragments were visualized under a 302-nm ultraviolet light, after staining with ethidium bromide. Digested PCR products for *fimA* type Ib were fragments of 162 and 109 bp. PCR detection limits were in the range of 25-100 cells (12,18). In the present study, the sensitivity of the PCR assay using the *fimA* type primer set was approximately 25-100 cells in plaque samples from the subjects, and PCR analysis using 16S rDNA primer confirmed the presence of bacteria (data not shown).

Statistical analysis

Chi-squared test was used for statistical analysis of the comparative frequencies of bacterial occurrence among all periodontal groups. Statistical analyses were conducted using SPSS 10.0J (SPSS Inc., Chicago, IL, USA).

Results

Values showing the distribution of *P. gingivalis* in children in the healthy gingiva, gingivitis and periodontitis groups, as well as the ratios for probability of *P. gingivalis* detection, are shown in Table 2. Overall, *P*. gingivalis was detected in 2 (1.5%) of 134 subjects in the healthy group, 24 (10.0%) of 239 in the gingivitis group, and 8 (29.6%) of 27 in the periodontitis group. Significant differences were observed in *P. gingivalis* infection among the groups [gingivitis group vs. healthy group, $P < 0.01$, odds ratio (OR) = 7.4; periodontitis group vs. healthy group, $P < 0.001$, OR = 27.8].

The distributions of *P*. *gingivalis* in the healthy, gingivitis and periodontitis groups in different dentition stages are shown in Table 3. In the gingivitis group, *P. gingivalis* was detected in 17 (11.8%) of 152 subjects with mixed dentition and 5 (11.6%) of 43 with permanent dentition, while it was found in 2 (4.5%) of 44 with primary dentition. In the periodontitis group, *P. gingivalis* was detected in 4 (25.0%) of 16 subjects with mixed dentition and 3 (75.0%) of 4 with permanent dentition, while it was found in 1 (14.3%) of 7 with primary dentition. In contrast, *P. gingivalis* was found in only 2 (4.3%) of 47 subjects in the healthy group with mixed dentition, while the organism was not isolated from any of the healthy subjects with either primary or permanent dentition.

The distribution of *fimA* genotypes among *P. gingivalis* organisms found in the plaque samples was subsequently examined. Table 4 shows the distribution of the 6 *fimA*

Table 4 Distribution of 6 *fimA* types in children possessing *P. gingivalis* divided into healthy, gingivitis, and periodontitis groups

	Percent frequency of occurrence (No. of positive subjects)			
fimA type	Healthy (2)	Gingivitis (24)	Periodontitis (8)	Total (34)
I	0	0	0	0
Ib	0	0	0	0
II	50.0 (1)	29.2 (7)	25.0 (2)	29.4 (10)
III	0	8.3 (2)	0	5.9 (2)
IV	0	33.3 (8)	37.5 (3)	32.4 (11)
V	0	0	0	0
Ib+II	50.0 (1)	16.7 (4)	37.5 (3)	23.5 (8)
Ib+II+V	0	4.2 (1)	0	2.9 (1)
Untypeable	0	8.3 (2)	0	5.9 (2)

Table 5 Distribution of 6 *fimA* types in children possessing *P. gingivalis* divided into different dentition stages

	Percent frequency of occurrence (No. of positive subjects)			
	Dentition			
fimA type	Primary (3)	Mixed (23)	Permanent (8)	Total (34)
I	0	0	0	0
Ib	0	0	0	0
II	66.7 (2)	21.7 (5)	37.5 (3)	29.4 (10)
III	0	8.7 (2)	0	5.9 (2)
IV	33.3 (1)	30.4 (7)	37.5 (3)	32.4 (11)
V	0	0	0	0
Ib+II	0	26.1 (6)	25.0 (2)	23.5 (8)
Ib+II+V	0	4.4 (1)	0	2.9 (1)
Untypeable	0	8.7 (2)	0	5.9 (2)

types in subjects in the healthy, gingivitis and periodontitis groups who possessed *P. gingivalis*. Overall, a single *fimA* genotype was detected in 67.7% of all subjects, with type IV most frequently detected (32.4%), followed by type II (29.4%). Multiple *fimA* genotypes were detected in 26.4% of all subjects. Among all *P. gingivalis*-positive periodontitis subjects, the most prevalent *fimA* types were type Ib/type II combination (37.5%) and type IV (37.5%), followed by type II (25.0%). Subjects with gingivitis predominantly possessed type IV (33.3%) and type II (29.2%). In contrast, type I was not detected in any of the subjects, while the type Ib was also not detected as a single *fimA* genotype. Untypeable strains were detected in 5.9% of all subjects, while type Ib was not detected in any subjects with primary dentition. Table 5 shows the distribution of the 6 *fimA* types in children possessing *P. gingivalis* divided into different dentition stages. Type II and type IV were detected in subjects with all types of dentition, while type Ib/II combination was only detected in subjects with mixed and permanent dentition.

Discussion

We investigated the prevalence of *P. gingivalis* in 400 children divided into healthy, gingivitis and periodontitis groups, with the pathogen detected in 1.5%, 10.0% and 29.6% of these subjects, respectively. Mättö et al. (20) reported that the detection frequency of *P. gingivalis* was 5% in saliva samples from subjects aged 5 to 10 years and 14% in those from subjects aged 11 to 20. We previously found that *P. gingivalis* was rarely present in the oral cavities of healthy children aged 2 to 12 years (5), of whom 4.8% of the subjects (0% with primary dentition and 11.1% with mixed dentition) were positive for *P. gingivalis* in plaque samples obtained by toothbrushing. Tamura et al. (14) also reported that samples from 3.2% of children and adolescents without periodontitis showed a positive reaction to *P. gingivalis*-specific primers, while Ashimoto et al. (18) found that the prevalence of

P. gingivalis in children aged 2 to 11 years with pediatric gingivitis was 14%. In the present study, we used a PCR method and detected *P. gingivalis* in children aged 2 to 15 years, and our results agreed with those of previous reports (5,14,18). Furthermore, the prevalence of *P. gingivalis* was significantly different among the present healthy, gingivitis and periodontitis groups, and we found evidence that the pathogen may be directly related to periodontitis in children. When we divided the periodontitis group based on stage of dentition, those with permanent dentition had the highest rate (75.0%) of occurrence, as compared to those with mixed (25.0%) and primary (14.3%) dentition. Our findings also agree with previous studies that reported a significant correlation between age and rate of detection of *P. gingivalis* (14,21).

In the present study, type Ib/type II *fimA* combination (37.5%) and type IV (37.5%) were predominant in children with periodontitis, followed by type II (25.0%). Nakagawa et al. (11) reported that type Ib/type II *fimA* combination was not present in any adults tested. Thus, the prevalence of this *P. gingivalis fimA* combination in children may be different from that in adults based on periodontal condition. Tamura et al. (14) later noted that none of their subjects were type II *fimA* positive. However, we found that 10 of 34 (29.4%) of our subjects who possessed *P. gingivalis* were type II *fimA*-positive. According to Missailidis et al. (22) and Miura et al. (23), the most prevalent *fimA* type in adult periodontitis patients is type II, followed by type Ib. In addition, Amano (8) reported that the majority of adult periodontitis patients harbor type II and that the next most prevalent is type IV. These results for adult periodontitis patients were similar to the present results obtained in children. We also reported previously that the prevalence of *P. gingivalis* was 71.4% in both fathers and mothers of proband families, while it was 35.7% in their children (24), and we found that parents are an important source of periodontal pathogens that colonize the oral cavities of their children (24). On the other hand, *P. gingivalis* was shown to be less easily transmitted from parents to children (25), while another study noted that the pathogen may be difficult to transmit or require a longer period of time for colonization (26). Asano et al. (27) also suggested that *fimA* type II, widely distributed in patients with periodontitis, may be an important factor in the transmission of *P. gingivalis* between spouses.

In the present *P. gingivalis*-positive gingivitis subjects, the most prevalent *fimA* type was type IV (33.3%), followed by type II (29.2%). In a study conducted in Brazil, Missailidis et al. (22) reported that those with gingivitis commonly had type I/type IV *fimA* combination, followed by subjects with type IV. It is possible that type IV and type II are related to gingivitis, as well as periodontitis, in children.

Several studies have shown that the most prevalent *fimA* genotype in healthy adults is type I (8,12,13,23), while organisms with types II and IV were found to be prevalent in only 9.4% and 6.5%, respectively, of studied subjects (13). However, in the present study, type I was not detected in any of the subjects, and type Ib and type V were not detected as a single *fimA* genotype. Nevertheless, longitudinal studies are required to evaluate the risk of occurrence and development of periodontitis in children.

In our study, untypeable *fimA* was found in 2 children (8.3%) with gingivitis, but not in any subjects with healthy gingiva or periodontitis. Tamura et al. (14) noted that one of the major characteristics of *fimA* genotype distribution in children and adolescents is a high frequency of untypeable strains (33.3%). On the other hand, Nakagawa et al. (11) reported that untypeable strains were found in adult subjects with (1.5%) and without (4.3%) periodontitis. Furthermore, Asano et al. (27) reported that untypeable strains were found in 6.3% of the adults with periodontitis. The discrepancies among these studies might be due, in part, to the sampling methods employed. However, further studies are required in order to better understand the virulence of untypeable *fimA*.

In summary, our findings indicate that the presence of *P. gingivalis* is associated with periodontal diseases, and that the type II, IV and Ib/II combination are the most common among *fimA* genotypes.

Acknowledgments

We would like to thank Dr. Makoto Kawamura of Hiroshima University Hospital for his advice regarding statistical methods. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (16791287).

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