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Distribution of *Porphyromonas gingivalis* Strains with *fimA* Genotypes in Periodontitis Patients

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Fimbriae (FimA) of *Porphyromonas gingivalis* are filamentous components on the cell surface and are thought to play an important role in the colonization and invasion of periodontal tissues. We previously demonstrated that *fimA* can be classified into four variants (types I to IV) on the basis of the nucleotide sequences of the *fimA* gene. In the present study, we attempted to detect the four different *fimA* genes in saliva and plaque samples isolated from patients with periodontitis using the PCR method. Four sets of *fimA* type-specific primers were designed for the PCR assay. These primers selectively amplified 392-bp (type I), 257-bp (type II), 247-bp (type III), and 251-bp (type IV) DNA fragments of the *fimA* gene. Positive PCR results were observed with reference strains of *P. gingivalis* in a type-specific manner. All other laboratory strains of oral and nonoral bacteria gave negative results. The sensitivity of the PCR assay for *fimA* type-specific detection was between 5 and 50 cells of *P. gingivalis*. Clinical samples were obtained from saliva and subgingival plaque from deep pockets (≥4 mm) of 93 patients with periodontitis. Bacterial genomic DNA was isolated from the samples, and the targeted fragments were amplified by PCR. The presence of *P. gingivalis* was demonstrated in 73 patients (78.5%), and a single *fimA* gene was detected in most patients. The distribution of the four *fimA* types among the *P. gingivalis*-positive patients was as follows: type I, 5.4%; type II, 58.9%; type III, 6.8%; type IV, 12.3%; types I and II, 6.8%; types II and IV, 2.7%; and untypeable, 6.8%. *P. gingivalis* with type II *fimA* was detected more frequently in the deeper pockets, and a significant difference of the occurrence was observed between shallow (4 mm) and deep (≥8 mm) pockets. These results suggest that *P. gingivalis* strains that possess type II *fimA* are significantly more predominant in periodontitis patients, and we speculate that these organisms are involved in the destructive progression of periodontal diseases.

Porphyromonas gingivalis is a gram-negative, black-pigmented anaerobe associated with several periodontal diseases including adult periodontitis, generalized juvenile periodontitis, periodontal abscesses, and refractory periodontitis (5, 31). This bacterium has most frequently been detected in deep periodontal pockets and has exhibited a low prevalence in healthy periodontal tissues without destructive inflammation (3, 10, 26).

It has become clear that heterogeneity exists in terms of virulence among various *P. gingivalis* strains, as assayed in experimental model systems. The encapsulated cells, which induced phlegmonous abscess and/or the necrosis frequently associated with death in experimental animals, are called virulent or invasive strains, and the nonencapsulated cells that induce pus formation and/or localized abscesses are classified into nonvirulent or noninvasive strains in animal models of subcutaneous infection (9, 24, 27, 29). It should be noted, however, that contradictory findings were obtained in another model with orally infected rats (7, 13). The encapsulated strains were less pathogenic than the noncapsulated strains. The animal model of subcutaneous infection measures the tissue invasiveness of the organisms, while the other model evaluates the capability of the organisms to attach to and colonize tissues in the mouth. Little information regarding the bacterial factors that determine the prevalence and distribution of *P. gingivalis* in patients with marginal periodontitis is available.

Studies have been performed to determine the distribution of the specific serotypes of *P. gingivalis* strains. In 63 periodontitis patients, all isolates from periodontal pockets were serotypeable with antisera raised against four strains representative of each type and 79.3% of the isolates reacted with the sera against their "type I" nonencapsulated strains (21). Other investigators used capsular K antigens for the serological typing of *P. gingivalis*. They showed that 45.4% of 185 strains from 185 patients were six K typeable (K1 to K6) and that K5 and K6 were predominant (14).

P. gingivalis fimbriae (FimA) are filamentous components on the cell surface and are thought to play an important role in the colonization and invasion of periodontal tissues (11, 19, 25). We previously demonstrated that the *fimA* gene can be classified into four variants (types I to IV) on the basis of the nucleotide sequences of the *fimA* gene (8, 12). It has been reported that strains 381, ATCC 33277, and HG565 expressing type I fimbriae strongly adhere to host proteins (2, 22, 23). The characterization of *P. gingivalis* fimbriae has been performed biochemically, genetically, and immunologically; however, nearly all of these studies were done with type I FimA (*fimA*) only (11, 25). The prevalence of strains that possess type I FimA (*fimA*) in humans is unknown. In this study, we developed a PCR assay to detect the four types of the *fimA* gene of *P. gingivalis* in saliva and plaque samples from patients, and we successfully identified the most predominant *fimA* type.

MATERIALS AND METHODS

Bacterial strains. *P. gingivalis* strains that possessed each of four *fimA* genotypes (12) were selected from our culture collections as follows: type I *fimA*, strains 381, BH18/10, and ATCC 33277; type II *fimA*, strains HW24D-1,

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TABLE 1. *fimA* type-specific or 16S rRNA-specific primers used in this study

Primer set	Sequence (5′ to 3′)	Size (bp)[a]
Universal primers for positive control	AGA GTT TGA TCC TGG CTC AG GGC TAC CTT GTT ACG ACT T	3,480
P. gingivalis 16S rRNA	TGT AGA TGA CTG ATG GTG AAA ACC ACG TCA TCC CCA CCT TCC TC	197
Type I *fimA*	CTG TGT GTT TAT GGC AAA CTT C AAC CCC GCT CCC TGT ATT CCG A	392
Type II *fimA*	ACA ACT ATA CTT ATG ACA ATG G AAC CCC GCT CCC TGT ATT CCG A	257
Type III *fimA*	ATT ACA CCT ACA CAG GTG AGG C AAC CCC GCT CCC TGT ATT CCG A	247
Type IV *fimA*	CTA TTC AGG TGC TAT TAC CCA A AAC CCC GCT CCC TGT ATT CCG A	251

[a] Expected size of PCR product.

OMZ314, and OMZ409; type III *fimA*, strains 6/26 and ATCC 49417; and type IV *fimA*, strains HG564 and W50. *P. gingivalis* strains representative of six different capsular serotypes (K types) (14, 30) were also used, i.e., strains W83 (K1), HG184 (K2), A7A1-28 (K3), ATCC 49417 (K4), HG1690 (K5), and HG1691 (K6). Organisms were grown in brain heart infusion broth as described previously (1). The specificities of the primers used were tested against the following organisms: *Fusobacterium nucleatum* ATCC 10953, *Actinobacillus actinomycetemcomitans* Y4, *Prevotella intermedia* ATCC 25261, *Streptococcus mutans* MT8148, *Streptococcus sanguis* ATCC 10556, and *Escherichia coli* NM522.

Clinical specimens. The subjects were 93 systemically healthy Japanese adults who had marginal periodontitis associated with periodontal pocket formations deeper than 4 mm (33 males aged 19 to 74 years [mean age, 55.6 ± 11.5 years] and 60 females aged 21 to 79 years [mean age, 54.6 ± 14.8 years]) and who were referred to the Osaka University Dental Hospital for dental or periodontal treatment. The subjects had received neither professional cleaning nor an antibiotic medication within the 3 months before the study. The subjects were enrolled with informed consent. Subgingival plaque samples were taken from mesial and lingual subgingival sites of all molars with sterile Gracy curettes after supragingival plaque was gently removed. The samples were placed in sterile tubes containing 1 ml of phosphate-buffered saline (pH 7.4) on ice. The pocket probing depth was subsequently measured. The specimens from the two sites with the deepest probing depths were selected and were then vortex mixed and centrifuged at 12,000 × *g* for 1 min to pellet the bacterial cells. The bacterial genomic DNA was isolated from the samples with a DNA isolation kit (Puregene; Gentra Systems, Minneapolis, Minn.) according to the manufacturer's instructions, and the isolated DNA was dissolved in 100 μl of distilled water. Expectorated whole saliva (ca. 1 ml) was also collected from each subject and placed into a sterile plastic tube on ice. The saliva samples were processed for the PCR assay by the method reported by Mättö et al. (20).

PCR primers and amplification. Table 1 lists the PCR primers designed for this study. The *fimA* genotype-specific forward primers were selected from type-specific segments of nucleotide sequences of the four genotypes, and the reverse primer was common for all of the *fimA* types as a conserved and *fimA*-specific sequence. The specificities of the prospective primers were tested by the program Amplify (6), based on the DNA sequence information stored in GenBank-EMBL. A ubiquitous primer set that matches almost all bacterial 16S rRNA genes was used as a positive control (4), and *P. gingivalis* species-specific primers (16S rRNA specific) were used as described previously (28). The specificities and sensitivities of the two primer sets described above for the target organisms were investigated in the original studies. All of the primers were commercially synthesized (Amersham Pharmacia Biotech, Uppsala, Sweden).

The PCR amplification was performed in a total volume of 25 μl consisting of PCR beads (Ready-To-Go; Amersham Pharmacia Biotech), 0.8 μM each primer, and 2 to 5 μl of the template DNA solution (20 to 60 μg/ml) in sterile distilled water. The amplification reaction was performed in a thermal cycler (model 2400; Applied Biosystems, Branchburg, N.J.) with the following cycling parameters: an initial denaturation at 95°C for 5 min, 30 cycles consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. Positive and negative controls were included in each PCR set and in the processing of all samples.

The PCR products were subjected to electrophoresis in a 2% agarose gel–Tris-acetate-EDTA buffer. The gel was stained with 0.5 μg of ethidium bromide per ml and photographed under UV illumination. A 100-bp DNA ladder (New England Biolabs, Beverly, MA) was used as a molecular size standard. The sensitivity of the PCR assay was studied with titrated cultures of *P. gingivalis* of the four *fimA* types (types I to IV; strains 381, HW24D-1, 6/26, and HG564, respectively; 10^9 cells/ml). The detection limit was determined for the simultaneous PCR by the use of known numbers of bacterial cells diluted with distilled water.

Periodontal examination. The clinical parameters were measured by a single skilled examiner (A.A.) and included the pocket probing depth and bleeding on probing. The pocket probing depth was measured to the nearest millimeter at six points on the circumference of each tooth (mesio-, mid-, and distobuccal and disto-, mid-, and mesiolingual) from the gingival margin to the deepest probeable point with a round-ended probe tip (diameter, 0.4 mm).

Statistical analysis. The chi-square test was used for the statistical analysis of the comparative frequencies of occurrence of the bacteria.

RESULTS

Specificity and sensitivity of the PCR assay. The *fimA* type-specific PCR products were selectively detected among strains of *P. gingivalis* and other species, as shown in Table 2. The positive PCR gave a single band with the expected sizes, as

TABLE 2. Specificities of primers against various target strains

Strain	Amplification with the following primer[a]:					
	I	II	III	IV	Pg	U
P. gingivalis						
381	+	−	−	−	+	+
ATCC 33277	+	−	−	−	+	+
BH18/10	+	−	−	−	+	+
HW24D-1	−	+	−	−	+	+
OMZ314	−	+	−	−	+	+
OMZ409	−	+	−	−	+	+
ATCC 49417	−	−	+	−	+	+
6/26	−	−	+	−	+	+
HG564	−	−	−	+	+	+
W50	−	−	−	+	+	+
A. actinomycetemcomitans Y4	−	−	−	−	−	+
F. nucleatum ATCC 10953	−	−	−	−	−	+
P. intermedia ATCC 25611	−	−	−	−	−	+
S. mutans MT8148	−	−	−	−	−	+
S. sanguis ATCC 10556	−	−	−	−	−	+
E. coli NM522	−	−	−	−	−	+

[a] +, positive amplification; −, no PCR product; I to IV, *fimA* types; Pg, *P. gingivalis* species-specific 16S rRNA primers; U, universal primers for positive control.

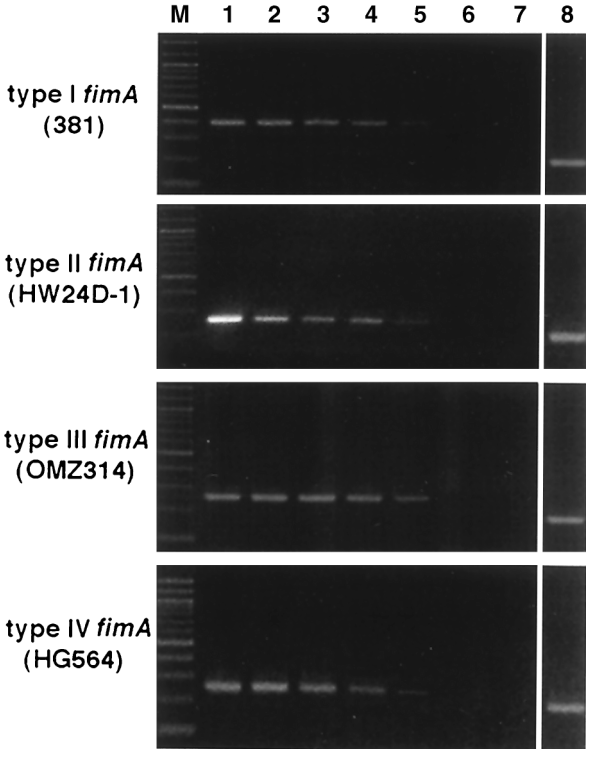

FIG. 1. Sensitivity of PCR assay for detection of our *fimA* types of *P. gingivalis*. The sensitivity of the PCR assay was studied with titrated cultures of *P. gingivalis* of four *fimA* types (types I to IV; 10^9 cells of strains 381, HW24D-1, OMZ314, and HG564, respectively, per ml). The detection limit was determined for the simultaneous PCR by the use of known numbers of bacterial cells diluted in sterile distilled water. The following numbers of cells were added: 5×10^5 (lane 1), 5×10^4 (lane 2), 5×10^3 (lane 3), 5×10^2 (lane 4), 5×10 (lane 5), and 5 (lane 6). Lane 7, a negative control from the PCR without any bacterial cells; lane 8, a PCR product obtained with *P. gingivalis* species-specific 16S rRNA primers (5×10^3 cells); lane M, molecular size marker (a 100-bp DNA ladder).

assessed by electrophoresis. No amplification was detected for any of the strains other than the prospective positive strains. The detection limit of the PCR assay was determined in the presence of titrated bacterial cells. As shown in Fig. 1, the PCR products were obtained as clear bands with the use of 50 cells in all *fimA* typings. No band was found in the lanes of PCR products with five or fewer cells including the negative control. Thus, the sensitivity of the PCR assay was shown to be between 5 and 50 cells for all *fimA* types. The sensitivities of the other two primer sets used for positive controls were in the same range as that of the *fimA*-specific primer (data not shown). No reaction was inhibited, even in the presence of 10^5 bacterial cells in a reaction mixture.

Detection of four *fimA* types of *P. gingivalis* in clinical samples. All six of the primer sets were simultaneously subjected to the PCR assay for each clinical specimen. The plaque and/or saliva samples of all 93 patients were positive for universal primers, and positive PCR results for *P. gingivalis* 16S rRNA primers were observed for 71 plaque samples and 66 saliva samples from a total of 73 patients (78.5% of the total subjects).

The distributions of the four *fimA* types among the 71 *P. gingivalis*-positive patients were successfully detected from plaque and saliva samples by the primers. A single *fimA* gene was detected in most of the samples, and the identical type was obtained with both plaque and saliva samples from a single subject. As shown in Table 3, type II *fimA* was detected at the highest frequency (58.9%) in the patients, and types I, III, and IV were found at very low frequencies of 5.4, 6.8, and 12.3%, respectively. In samples from seven patients, two different *fimA* types were simultaneously detected, i.e., five (6.8%) patients were each infected with types I and II and 2 (2.7%) patients were each infected with types II and IV. Five samples (6.8%) gave a negative PCR result with all of the *fimA*-specific primers (untypeable). The total incidence of type II *fimA* was 68.4%, indicating that more than two-thirds of the *P. gingivalis*-positive patients harbored type II *fimA* organisms. The four sets of type-specific *fimA* primers provided no positive PCR result for the samples which were negative for *P. gingivalis* with 16S rRNA primers. This result supports the specificity of the *fimA* primers.

TABLE 3. Distribution of four *fimA* types among *P. gingivalis*-positive patients: relationship to pocket depth and age-gender

Parameter (no. of patients[a])	Frequency of occurrence (%) of the following *fimA* type[b]:						
	I	II	III	IV	I and II	II and IV	UT[c]
P. gingivalis-positive patients (73)	5.4	58.9	6.8	12.3	6.8	2.7	6.8
Pocket probing depth (mm)							
4 (9)	22.2	44.4	0	11.1	0	0	22.2
5 (19)	5.2	52.6	10.5	15.8	5.3	5.3	5.3
6 (22)	0	54.5	13.6	9.1	9.1	4.6	9.1
7 (12)	0	66.7	0	16.6	16.6	0	0
8–15 (11)	9.1	90.9	0	0	0	0	0
Age (yrs)							
Male							
19–35 (3)	0	33.3	33.3	0	0	0	33.3
36–57 (14)	0	57.1	14.2	21.4	7.1	0	0
58–74 (13)	0	53.8	7.7	15.4	7.7	0	15.4
Female							
24–35 (6)	0	33.3	16.7	16.7	16.7	16.7	0
36–57 (18)	11.1	83.3	0	5.6	0	0	0
58–79 (19)	10.5	52.6	0	10.5	10.5	5.3	10.5

[a] Number of *P. gingivalis*-positive patients.
[b] Significant differences ($P < 0.05$) were found for the following comparisons: type II versus type I, III, IV, I-II, and II-IV, and untypeable infections; 4-mm versus 8- to 15-mm pocket probing depth male versus female sex; and age 24 to 35 years versus age 36 to 57 years for females.
[c] UT, untypeable.

Relationship of *fimA* types to clinical parameters. The relationship of some clinical parameters to the prevalence of *fimA* types was analyzed (Table 3). A linear relation was found between the pocket probing depth and the prevalence of type II *fimA* organisms, and a significant difference ($P < 0.05$) was obtained between the occurrence of type II organisms in shallow (4 mm) and deep (≥8 mm) pockets. In addition, the other *fimA* types seemed to occur more often in the pockets with shallow and moderate depths. The relationships between age/gender and the distribution of *fimA* types were also analyzed (Table 3). *fimA* type I was exclusively detected in female subjects ($P < 0.05$). The incidence of *fimA* type II was slightly greater in the middle age group (age, 36 to 57 years) than the other age groups for both the males and females, yet, a significant difference was observed only between the younger and middle age groups for females ($P < 0.05$).

Bleeding on probing was found in 77 patients (86%). There was no clear relationship between the patients with bleeding on probing and the prevalence of *fimA*.

***fimA* types of K-serotypeable strains.** The representative K-serotype strains were used in a PCR assay to determine their *fimA* types. Most of the K-typeable strains (the exceptions were the K1 and K6 strains) were shown to possess type II *fimA*, as follows: W83 (K1), type IV *fimA*; HG184 (K2), type II; A7A1-28 (K3), type II; ATCC 49417 (K4), type II; HG1690 (K5), type II; and HG1691 (K6), untypeable.

DISCUSSION

This is the first report of an investigation on the prevalence of *P. gingivalis fimA* genotypes in saliva and plaque samples from periodontitis patients. The present assay with the designed *fimA* type-specific primer sets gave clear PCR products, and the assay had sufficient sensitivities and specificities. The results obtained in the present study demonstrate that *P. gingivalis* strains that possess the type II *fimA* gene are most predominantly present in the oral flora of the periodontitis patients and that the type II *fimA* organisms might be involved in the etiology of advanced periodontitis. To date, only limited numbers of *P. gingivalis* strains have been shown to possess type II *fimA*, e.g., strains HW24D1, OMZ314, OMZ409, A7A2-10, AJW4, THUR28BM2, AJW3, JKG7, and A7A1-28 (ATCC 53977) (12, 16). A wide variety of studies regarding the adhesive functions and immunobiological activities of fimbriae have been performed (11). These studies suggested that *P. gingivalis* fimbriae are major virulence factors and are possible candidates for use in a vaccine. However, most investigators have dealt with type I FimA (*fimA*) in their studies; few studies with other types of fimbriae have been performed. Although the four types of fimbriae were previously purified from the organisms (15), no characterization of the functional heterogeneity of fimbriae has been reported yet. It should be noted that type II *fimA* strain A7A1-28 reportedly induced necrosis with death in an animal model (24), and strains of the virulent K types (types K2 to K5) also possess type II *fimA*, as revealed in this study. The virulent and invasive strains are all encapsulated, and these encapsulated strains were reported to be less adhesive to host proteins such as collagen types I and IV, fibronectin, laminin, and salivary proteins (2, 22, 23). To clarify the ecological factors that determine the colonization ability of *P. gingivalis* in humans, the characterization of the most prevalent type of fimbriae is required. It is also necessary to determine whether various type II *fimA* strains in the patients are virulent in animal models.

The prevalence and distribution of six K-typeable strains were previously examined by Laine et al. (14) in periodontitis patients, and the following prevalence ratios were reported: K1, 3.8%; K2, 2.2%; K3, 1.1%; K4, 3.2%; K5, 12.0%; and K6, 23.2%. The combined findings of their report and our own results suggest that type II *fimA P. gingivalis* may carry other antigens. Another serological study was performed with four different antisera against the reference strains to investigate the prevalence of serotypes of *P. gingivalis* in periodontitis patients (21). All of the isolated *P. gingivalis* strains were typeable as one of these four serotypes; 44.4% serotype I strains were reactive with anti-strain 381 serum, 11.1% serotype II strains were reactive with anti-JH4 serum, 6.3% serotype III strains were reactive with anti-W50 or anti-W83 serum, and 34.9% serotype IV strains were reactive with anti-ATCC 33277 serum. The serotype I and IV strains in that study possessed type I *fimA*, while the serotype III strains had type IV *fimA*. The *fimA* type of the serotype II strains was unknown. These results indicate that type I *fimA* strains are more frequently detected in periodontitis patients, which is not in agreement with our present findings. These serological studies were performed by the culture technique, and the method is not sensitive enough. The PCR assay can be expected to be more sensitive and specific than the culture method, as reported previously (4, 17).

Our present findings indicated the involvement of type II *fimA* strains in advanced periodontitis. The type II strains are also predominant in the middle age group, especially in females. Thus, it might be possible that the periodontitis patients infected or colonized with type II *fimA* strains could lose their dentition earlier due to the destructive nature of the harbored strains compared to the time to the loss of dentition for patients infected or colonized with other *fimA* type strains.

Five of the patients in the present study were shown to harbor *fimA* untypeable strains, indicating that more than four genotypes may exist in *P. gingivalis*. A restriction fragment length polymorphism (RFLP) analysis showed that the majority of *fimA* loci of 38 *P. gingivalis* strains could be classified into four groups, and the other seven strains were divided into three minor groups with Southern blots probed with *fimA* of strain 381 (18). Although those results may reflect the diversities of *fimA* flanking regions, they might also support the existence of other *fimA* genotypes. The *P. gingivalis* strains divided in the major four RFLP groups probably correspond to those classified by present *fimA* genotypes I to IV. The minor three RFLP groups might indicate the unknown *fimA* types. The cloning and sequencing of novel *fimA* genes isolated from the untypeable samples are under investigation in our laboratories.

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