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Quantification of *Porphyromonas gingivalis* and *fimA* genotypes in smoker chronic periodontitis

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Abstract

Aim: *Porphyromonas gingivalis fim*A genotypes were associated with virulence factors *in vitro*, but little evidence of an association with disease severity were shown in humans. We aimed to correlate levels of *P. gingivalis fimA* genotypes II and IV and probing depth in smoker-chronic periodontitis subjects.

Material and Methods: One hundred and sixty eight subgingival samples of 20 smokers non-treated chronic periodontitis subjects obtained from sites with different probing depths [shallow ($\leqslant$3 mm), intermediate (4–6 mm), deep ($\geqslant$7 mm)] were analysed by real-time PCR for *P. gingivalis* and genotypes *fimA* II and IV.

Results: *P. gingivalis* and *fimA* IV were detected in all subjects, whereas *fimA* II was detected in 18 subjects (90%). One hundred and fifty two sites (90.5%) harboured *P. gingivalis*. Genotypes II and IV were detected in 28% and 69.6% of sites, respectively. The proportions of genotypes II and IV in relation to *P. gingivalis* levels were similar in shallow, intermediate and deep probing sites (2.4%, 4.6%, 1.4% for genotype II and 15.5%, 17.7%, 11.7% for genotype IV, respectively), indicating that other non-tested genotypes were more abundant. Increased levels of genotype IV were associated with increasing probing depth, but not of genotype II.

Conclusions: The data suggested an association between *P. gingivalis* genotype *fimA* IV and disease severity in smoker-chronic periodontitis subjects.

Key words: *fimA* genotypes; *Porphyromonas gingivalis*; real time PCR; smokers; subgingival biofilm

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Porphyromonas gingivalis is a Gram-negative anaerobic coccobacillus associated with the aetiology of chronic periodontal diseases (Darveau et al. 1998). This microorganism exhibits genotypic and phenotypic diversity, which may result in differences in the ability of individual clones to induce periodontal destruction (Griffen et al. 1999, Amano et al. 2001).

P. gingivalis fimbriae are filamentous components located on the cell surface (Sugano et al. 2004) that are thought to play a significant role in the colonization of the oral cavity and invasion of periodontal tissues (Hiramine et al. 2003). *fimA*, encoding fimbrillin (a subunit protein of fimbriae), has been classified into six genotypes (types I, Ib, II, III, IV and V) based on the nucleotides sequences (Amano et al. 2004). Most periodontitis patients were found to harbour *P. gingivalis fimA* type II followed by type IV (Eick et al. 2002, Amano et al. 2004, Missailidis et al. 2004, van der Ploeg et al. 2004, Enersen et al. 2008). In contrast, type I was the most prevalent in healthy adults (Amano et al. 2000), indicating that there may be disease and non-disease associated *P. gingivalis* strains. Genotype *fimA* II strains degraded both paxillin and FAK more quickly than other *fimA* type strains, resulting in inhibition of phosphorylation by these molecules (Amano et al. 2004), and induced significantly higher levels of IL-8, MCP-1 and TNF-α than the other tested strains (Bodet et al. 2006). In addition, a recent study suggested the involvement of genotypes II and IV in the initiation and progression of cardiovascular diseases (Nakano et al. 2008). Beyond differences in prevalence, *fimA* genotypes of *P. gingivalis* seem to differ also in their ability to reach high cell densities in vivo (van der Ploeg et al. 2004), as well as in the transmission potential to other hosts (Asano et al. 2003).

In order to test the hypothesis that certain genotypes of *P. gingivalis* are

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more virulent than others, this study aimed to quantitatively evaluate the subgingival levels of *P. gingivalis fimA* genotypes II and IV in different probing categories in smoker-chronic periodontitis subjects by real time PCR.

Material and Methods

Study population

The study comprised 20 subjects, randomly selected, with chronic periodontitis (11 men, nine women, mean age 40.7 years, age range 30–52 years) who smoked $\geqslant 10$ cigarettes per day for at least 5 years (Ammenheuser et al. 1997) and were referred to receive periodontal treatment at Guarulhos University Dental School (Brazil). Inclusion criteria were $\geqslant 15$ natural teeth and a minimum of six teeth with at least one site non contiguous with pocket probing depth (PPD) between 5 and 7 mm and attachment loss between 5 and 10 mm. Exclusion criteria included pregnancy, previous periodontal treatment, history of systemic or local use of antimicrobials during the 6 months before sampling, or a known systemic condition that could influence the periodontal condition. Before sample collection, the subjects were requested to sign an informed consent to participate in the study. This study was approved by the Ethical Committees of the Institute of Biomedical Sciences, University of São Paulo and Guarulhos University.

Clinical measurements

Plaque accumulation (0/1), gingival bleeding (0/1), bleeding on probing (0/1), suppuration (0/1), probing pocket depth (mm) and clinical attachment level (mm) were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding third molars. These clinical parameters are summarized in Table 1.

Table 1. Mean (± SD) clinical parameters of subjects in the present study

	$N = 20$
Age (years)	40.7 ± 6.74
Gender (M/F)	11/9
Probing depth (mm)	3.92 ± 1.85
Attachment level (mm)	4.57 ± 1.95
% of sites with:	
Plaque	67.5 ± 21.0
Gingival bleeding	13.9 ± 21.5
Bleeding on probing	82.1 ± 20.7
Suppuration	1.8 ± 5.7

Microbial assessment

Plaque sample collection

After removal of supragingival plaque, subgingival plaque samples were taken using sterile Gracey curettes of nine sites per subject, three in each of the following categories: shallow ($\leqslant 3$ mm), intermediate (4–6 mm) and deep ($\geqslant 7$ mm) and placed in separate microtubes containing 100 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6) and stored at − 20°C until use.

Bacterial strains and growth conditions

P. gingivalis strains HW24D-1 and W83, used as a source of DNA for amplification of *fimA* alleles II and IV, respectively, were generously given by Dr. Atsuo Amano (Faculty of Dentistry, Osaka University, Japan). Strains were cultured anaerobically in Tryptone soy blood agar plates supplemented with haemin (0.5 mg/ml) and menadione (1 mg/ml) (Missailidis et al. 2004) for 7–15 days at 37°C in 85% N_2, 5% CO_2 e 10% H_2 in an anaerobic chamber (Plas Labs, Lansing, MI, USA).

Escherichia coli DH5α was used as host for plasmids and was grown aerobically at 37°C in LB medium (Umeda et al. 2006).

DNA isolation from plaque samples and bacterial reference cultures

Chromosomal DNA from cultures of *P. gingivalis* was isolated by boiling a bacterial suspension for 10 min. (Teanpaisan & Douglas 1999). Plaque samples were added to 20 μl TE containing 0.5% Tween 20 (Vetec, Rio de Janeiro, Brazil) and 200 μg/ml proteinase K (Sigma, St. Louis, MO, USA) and heated at 55°C for 2 h and 95°C for 5 min. in a termocycler (PTC-200 MJ Research, Waltham, MA, USA). The samples containing the DNA were stored at − 20°C until use.

Quantitative PCR

To establish the quantitative assay, plasmids containing the target genes were used as standards. PCR amplicons for *16SrRNA, fimA II* and *fimA IV* were obtained, individually cloned in pCR 2.1 TOPO TA (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations and transformed in *E. coli*. After growth of transformants, plasmids were extracted using PureLink Quick Plasmid Miniprep kit (Invitrogen) and sequenced.

Quantitative PCR was carried out using Quantimix Easy SYG Kit (Biotools, Madrid, Spain). PCR reactions were set up in 96-wells plates in a total volume of 20 μl containing 10 μl Sybr Green mix, 0.5 μl sample DNA and 200 mM of each primer for *16SrRNA* and *fimA II* and *fimA IV* (Amano et al. 1999, 2000). Temperature cycling profiles included a preincubation at 95°C for 10 min., followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 5 s and extension at 72°C for 20 s (van der Ploeg et al. 2004).

The detection of fluorescent products was monitored once every cycle selectively at 1°C bellow the melting point. Melting curves analysis were carried out in the range from 65 to 95°C to confirm that the PCR products from samples and reference plasmids had identical melting points.

Quantification of *P. gingivalis* and *fimA* copy numbers was obtained by comparison with standard curves with diluted plasmids harbouring a cloned copy of the template DNA. Plasmid standards and clinical samples were run in duplicates and average values were used for calculation of the bacterial load. Data were analysed using the software iQ5 Optical System Bio-Rad, (Hercules, CA, USA). The number of *P. gingivalis* cells was calculated by dividing the number of copies of *16SrRNA* by four, since *P. gingivalis* harbours four copies of this gene in the genome (Maeda et al. 2003), whereas each *fimA* copy corresponds to a single genome (Zhao et al. 2007).

As an additional control, quantitative analysis of *P. gingivalis* levels was performed in DNA samples obtained from suspensions of *P. gingivalis* strains HW24D-1 and W83 using the primers pairs *16SrRNA* and *fimAII and IV*.

Statistical analyses

Variance analysis using Friedman and Wilcoxon tests was used to assess differences in genotypes levels according to probing depths categories. The level of significance was set at 5% and the data were analysed using the software SPSS 13.0.

Results

All subjects presented at least one positive site for *P. gingivalis* and genotype *fimA* IV, while genotype *fimA* II was detected in 18 (90%) subjects. *P. gingivalis* was detected in 152 of 168 studied sites (90.5%), whereas *fimA II* was detected in 47 sites (28%) and *fimA IV* in 117 sites (69.6%).

Thus, 18 subjects (90%) harboured both genotypes, whereas genotype *fimA* IV was detected in the remaining two subjects. Forty-four sites (28.9%) exhibited both studied genotypes (*fimA* II and *fimA* IV). A single *fimA* variant was detected in 76 sites (50.0%), from which 73 (48.0%) sites were positive for *fimA* IV and only three (2.0%) sites were positive only for *fimA* II. The studied genotypes were not detected in 32 sites (21.1%) positive for *P. gingivalis*. There were no differences in the prevalence of *P. gingivalis* or genotypes II and IV according to probing depth (Fig. 1).

The mean counts of *P. gingivalis* were 578 cells/μl (5.78×10^4 cells/sample). In average, 13.7% of total *P. gingivalis* cells corresponded to genotype IV and only 2.6% to genotype II. Thus, 83.7% of *P. gingivalis* cells detected by RT-PCR belonged to other genotypes different from genotypes II and IV. Similar values of proportion of genotypes II and IV in relation to the levels of *P. gingivalis* were observed in shallow, intermediate and deep probing depth sites (15.5%, 17.7% and 11.7% for genotype IV and 2.4%, 4.6% and 1.4% for genotype II, respectively). The proportions of non-identified genotypes were also similar in shallow, intermediate or deep probing depths (82.1%, 77.7% and 86.9%, respectively) (Fig. 2).

The levels of *P. gingivalis* and genotypes *fimA* II and IV in relation to probing depth categories are shown in Fig. 3. *P. gingivalis* levels were higher in deep pockets than in shallow/intermediate ones. There were no differences in the mean levels of genotype II according to probing depth. However, the levels of genotype IV significantly increased with increasing probing depths.

Discussion

In the present study, *P. gingivalis* and *fimA* genotypes II and IV were quantified by real time PCR at the site level, in subgingival samples of 20 chronic periodontitits subjects. Determination of abundance at each site in sites with different probing depths was chosen in order to investigate if there was a site specific pattern of colonization by *P. gingivalis* and their *fimA* genotypes. *P. gingivalis* was detected in more than 90% of sites in all periodontitis subjects analysed, even in shallow sites (Fig. 1), indicating a need for its evaluation by quantitative methods. These same samples were tested in another study using Checkerboard DNA–DNA hybridization and all subjects were *P. gingivalis* positive (Matarazzo et al. 2008). Characteristics of the studied population such as the smoking habit and the geographical location may have contributed to the high prevalence and levels of *P. gingivalis* determined in this study. It was previously shown that Brazilian subjects with chronic periodontitis harbour higher levels of *P. gingivalis* when compared with other populations (Haffajee et al. 2004). In addition, smoking affects host response and contributes to periodontal disease progression (Grossi et al. 1996, Apatzidou et al. 2005, Bergström 2005, Palmer et al. 2005).

Although there are reports of an increased prevalence of *P. gingivalis* in smokers, due to the presence of deeper pockets (Bergström 2005), no differences in total counts or proportions of *P. gingivalis* are found in smokers when compared with non-smokers (Gomes et al. 2006). The smoking subjects were chosen since the habit may lead to additional loss of attachment even after therapy (Palmer et al. 1999), increasing the need for understanding the disease in such patients. In addition, the prevalence of *fimA* genotypes was reported by various studies with no distinction between subjects regarding to the smoking habit (Amano et al. 1999, Beikler et al. 2003, Missailidis et al. 2004).

P. gingivalis levels were higher in deep pockets than in sites with probing depth <6 mm, but they did not differ from shallow to intermediate depth sites. Previous studies using semi-quantitative analysis such as checkerboard DNA–DNA hybridization had shown that mean counts of *P. gingivalis* were higher in sites with probing depths >3 mm than in shallow gingival sulcus (Ximénez-Fyvie et al. 2000).

Genotypic diversity of *P. gingivalis* was assessed since a high prevalence of *P. gingivalis* was found in healthy sites in periodontitis subjects. Genotypes *fimA* II and *fimA* IV were selected due to their high prevalence among periodontitis patients, as well as reports of an increased virulence of these variants (Eick et al. 2002, Amano et al. 2004, van der Ploeg et al. 2004, Bodet et al. 2006, Enersen et al. 2008). In addition, prevalence of genotype II was correlated with deep pockets in Japanese periodontitis subjects (Amano et al. 1999).

The distribution of *fimA* genotypes was not homogenous in different subgingival sites in the same subject. Most studies analysing the prevalence of *P. gingivalis* genotypes, including our own previous study, analysed pooled plaque samples (Eick et al. 2002, Amano et al. 2004, Missailidis et al. 2004). A previous study which evaluated *P. gingivalis fimA* genotypes by quantification methods had also used pooled plaque samples, thus differences in genotypes distribution according to sites could not be determined (van der Ploeg et al. 2004).

In the present study, genotype *fimA* IV was detected in 69.6% of the sites, whereas *fimA* II was detected in only 28%. These data are in contrast with population studies in Japan, Brazil and Germany (Eick et al. 2002, Amano et al.

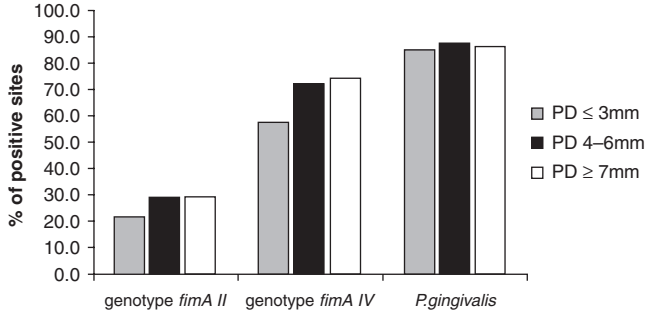

Fig. 1. Frequency of detection of *Porphyromonas gingivalis* and genotypes *fimA* II and *fimA* IV in 168 subgingival sites of 20 subjects with chronic periodontal disease according to probing depth categories.

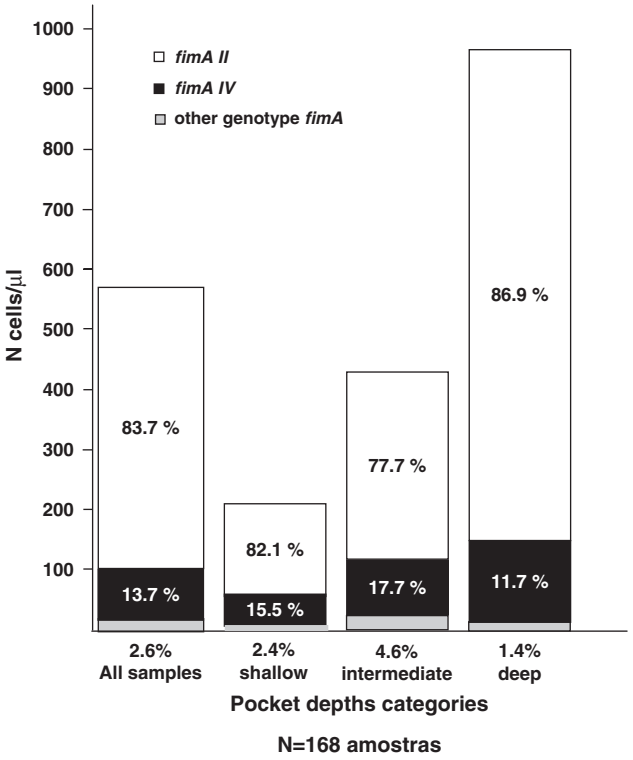

Fig. 2. Levels and proportion of genotypes *fimA* II and *fimA* IV in different pocket depth categories.

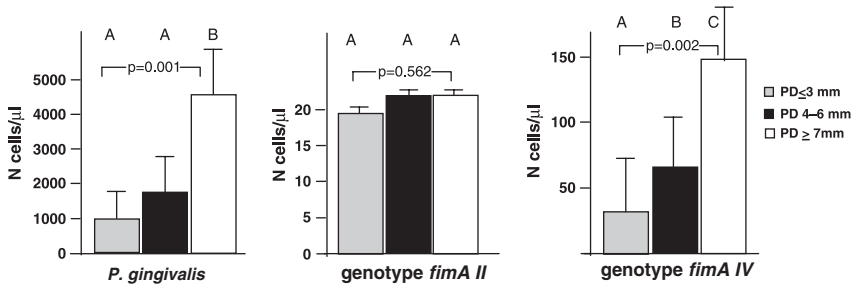

Fig. 3. Levels of *Porphyromonas gingivalis* and genotypes *fimA* II and *fimA* IV in subgingival sites of 20 subjects with chronic periodontal disease according to probing depth categories. #Significant difference among different probing depth categories, Friedman's test, $p < 0.001$. Different letters indicate significant difference between probing depth categories; Wilcoxon, $p < 0.05$.

2004, Missailidis et al. 2004, Enersen et al. 2008), but in agreement with analysis of deep pockets pooled samples from Norwegian and Swiss chronic periodontitis subjects by real-time PCR, which also revealed a higher prevalence of genotype *fimA* IV compared with *fimA* II (van der Ploeg et al. 2004).

The detection and proportions of genotypes II and IV in relation to total *P. gingivalis* counts were not correlated with clinical parameters of periodontal loss. However, a significant increase in levels of genotype *fimA IV* was associated with increasing probing depth, whereas levels of genotype *fimA* II did not change according to pocket probing categories (Fig. 3).

The low mean proportion of genotypes *fimA* II (2.6%) and *fimA* IV (13.7%) in relation to total *P. gingivalis* levels should be noted (Fig. 2). This result cannot be attributed to methodological errors, since the control using DNA from *P. gingivalis* strains with primers pairs for *16SrRNA* and *fimA* II or IV resulted in similar levels of cells (data not shown). Thus, our data clearly showed that other non-identified genotypes besides type II and IV were highly prevalent, and may have reached high proportions in the subgingival biofilm of the studied population. In a previous study, we have shown that the six known genotypes of *P. gingivalis* could not be detected in almost 20% of Brazilian periodontitis subjects harbouring the bacteria (Missailidis et al. 2004). The high prevalence of types I and Ib in the Brazilian population may have contributed in part to the non-identified genotypes. However, these genotypes were not investigated since *fimA* Ib shares a high degree of homology with *fimA* I and II (Enersen et al. 2008). Thus, the use of primers for type I would result also in detection of type Ib cells, which would have already been quantified in the reaction for type II, introducing an error in the quantitative analysis of *fimA* genotypes.

Genotypes III and V were also excluded since we have previously shown that these genotypes were very seldom found among Brazilians subjects (Missailidis et al. 2004, Umeda et al. 2006).

The data indicated that in smoker-chronic periodontitis Brazilian patients, genotype IV is not only more prevalent and abundant in periodontal pockets than genotype II, but its levels were associated with probing pocket depth. Genotype *fimA* I is usually associated with healthy sites, whereas genotype II is more associated with severe disease and possess more virulent traits than the others (Amano et al. 2000, Bodet et al. 2006). However, the phenotype of *fimA* II isolates is not homogeneous, at least regarding adherence and invasion properties (Umeda et al. 2006), and induction of inflammation in animal models (Inaba et al. 2008). In addition, it was recently shown by MLST (multi locus sequence typing) that *P. gingivalis* isolates from the same *fimA* genotype can be classified in several different clusters, although there was a tendency to cluster isolates with a certain *fimA* genotype in the same or closely related STs (Enersen et al. 2008). Other factors besides the fimbriae, such as gingipain production (Inaba et al. 2008), or genes encoded in a pathogenicity island (Curtis et al. 1999) seem to alter virulence in *P. gingivalis* isolates. Several strains belonging to genotype IV such as strains W50 and W83 are very well known for their pathogenic potential in inducing experimental abscess in animal models and in multiplying under iron limiting conditions (Grenier et al. 2001), but virulence factors specific for/or increased in *fimA* type IV strains are

still not known (Tachibana-Ono et al. 2008).

The association between *P. gingivalis* genotype *fimA* IV and disease severity in smoker-chronic periodontitis subjects indicated an increased bacterial challenge induced by this genotype. However, since a genotyping method based on the nucleotide sequence of *fimA* was used, these data do not suggest that FimA IV is the virulence factor involved in the possible increased virulence of genotype *fimA* IV. Despite, a single copy of *fimA* was reported in the genome of all tested *P. gingivalis* strains (Enersen et al. 2008), certain isolates, such as the highly virulent strain W83 (genotype *fimA* IV), do not express the fimbriae. On the other hand, strain W83 expresses a polysaccharide capsule, which may be involved in evading host immune response (Yoshimura et al. 2002). Thus, the observation that *fimA* type IV is more often found in sites with increased probing depth in chronic periodontitis subjects, supported by MLST data indicating some tendency for clustering of the *fimA* genotypes (Enersen et al. 2008), suggested that *fimA IV* genotype may belong to a lineage among the *P. gingivalis* population which express one or more key factors related to virulence.

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Clinical Relevance

Scientific rationale for the study: Although previous studies have associated *fimA* genotypes of *P. gingivalis* II and IV with periodontitis and these strains exhibited a higher virulence potential, these findings have not been correlated with clinical parameters of disease severity.

Principal findings: In smoker-chronic periodontitis Brazilian patients, genotype IV was not only more prevalent and abundant in periodontal pockets than genotype II, but its levels were associated with probing pocket depth.

Practical implications: The association between *P. gingivalis* genotype *fimA* IV and disease severity in smoker-chronic periodontitis subjects may have implications regarding prognosis and therapeutic approaches.