ELSEVIER

FEMS Microbiology Letters 232 (2004) 31–37

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Quantitative detection of *Porphyromonas gingivalis fimA* genotypes in dental plaque

Jan R. van der Ploeg [a], Elin Giertsen [b], Beat Lüdin [a], Christian Mörgeli [a], Annelies S. Zinkernagel [c], Rudolf Gmür [a,*]

[a] *Institute for Oral Biology, Section for Oral Microbiology and General Immunology, Center for Dental, Oral Medicine and Maxillofacial Surgery, University of Zürich, Plattenstrasse 11, CH-8028 Zürich, Switzerland*

[b] *Department of Odontology-Cariology, University of Bergen, Bergen, Norway*

[c] *Department of Medicine, University Hospital, University of Zürich, Zürich, Switzerland*

Received 17 September 2003; received in revised form 17 November 2003; accepted 5 January 2004

First published online 14 February 2004

Abstract

We developed quantitative *fimA* genotype assays and applied them in a pilot study investigating the fimbrial genotype distribution of *Porphyromonas gingivalis* in European subjects with or without chronic periodontitis. *P. gingivalis* was found in 71% and 9% of the samples from patients and healthy subjects, respectively. Enumeration of total *P. gingivalis* cell numbers by polymerase chain reaction and immunofluorescence showed excellent correspondence ($r = 0.964$). 73% of positive samples contained multiple *fimA* genotypes, but generally one genotype predominated by one to three orders of magnitude. Genotype II predominated in 60% of the samples. Genotype IV occurred with similar prevalence (73%) as genotype II but predominated in only 20% of the samples. Genotypes I, III and V were of much lower prevalence and cell densities of the latter two remained sparse. Our results suggest marked differences among the *fimA* genotypes' ability to colonize host sites with high cell numbers.

Keywords: Periodontitis; Bacterial fimbriae; *Porphyromonas gingivalis*

1. Introduction

Porphyromonas gingivalis is a Gram-negative anaerobic rod with deep human periodontal pockets as its preferred habitat. Due to its strong association with chronic periodontitis [1] and its wealth of potential virulence factors [2], the species is widely considered to be a major cause of chronic periodontitis in adults. *P. gingivalis* expresses thin fimbriae evenly distributed on the cell surface. There is ample evidence from in vitro and animal experiments that these fimbriae are important for binding to host cells and saliva-coated hydroxyapatite (see [2] for review). They also elicit a strong humoral host response [3] and it remains to be determined to what extent host antibodies interfere with fimbria-mediated adhesion. The major subunit of *P. gingivalis* fimbriae is FimA, a 43-kDa protein encoded by the *fimA* gene of which cells have a single copy [4]. Genetic analyses revealed *fimA* heterogeneity [5], reflected in the distinction of multiple genotypes [6,7]. Recent studies with Japanese subjects differing in periodontal health status revealed an exceptionally high prevalence (37%) of *P. gingivalis* among healthy individuals and an association of *fimA* genotype II with severe periodontitis, whereas periodontally healthy subjects mostly harbored genotype I [8]. These findings are of particular interest since the well-documented association of *P. gingivalis* with chronic periodontitis is not clearly understood. The species has a population structure characterized by frequent recombinations [9]. In general such a panmictic population structure is typical for opportunistic pathogens with a basically random distribution of potential virulence factors [10,11]. Amano et al.'s findings [8] seem to suggest, therefore, that *fimA* diversity represents an isolated trait that is highly associated with *P. gingivalis* virulence, and one would then expect that very similar *fimA* genotype distributions must be present in subject groups from other geographical regions.

* Corresponding author. Tel: +41 (1) 634 3306; Fax: +41 (1) 634 4310.
E-mail address: gmuer@zzmk.unizh.ch (R. Gmür).

0378-1097/04/$22.00
doi:10.1016/S0378-1097(04)00064-3

The aim of this study was to generate quantitative assays for the enumeration of the various *P. gingivalis* fimbrial subtypes and to monitor these cells in a pilot population of Swiss and Norwegian subjects with or without chronic periodontitis. To be able to quantitate even minor populations of *fimA* genotypes, a quantitative real-time polymerase chain reaction (PCR) assay was developed and employed.

2. Materials and methods

2.1. Study population

The studied cohort comprised 17 patients with chronic periodontitis (six men, 11 women, mean age 53.1 years, age range 38–81 years) and 12 periodontically healthy subjects (six men, six women, mean age 30.2 years, age range 22–46 years) from two periodontists with private offices in the Zürich area (Switzerland). In addition, 21 periodontically healthy adults (seven men, 14 women, mean age 23.4 years, age range 19–33 years) were selected among patients of the Department of Odontology from the University of Bergen (Norway). Inclusion criteria for subjects with chronic periodontitis were ≥ 20 natural teeth and ≥ 4 sites with ≥ 4 mm pocket probing depth (PPD) and attachment loss; the corresponding criteria for the healthy control group were ≥ 24 natural teeth and no sites with > 3 mm PPD or attachment loss. None of the participants was pregnant, had scaling or a history of systemic or local use of anti-infectiva during the 4 months prior to sampling, or a known systemic condition that could have influenced periodontal health. All subjects gave their written, informed consent to participating in this study, which was approved by the local region's ethical committee.

2.2. Periodontal examination and plaque sample collection

PPD and loss of attachment, verified by X-ray examinations, were determined to the nearest millimeter [12]. The four test sites had 4–10 mm (mean 6.4 ± 0.18 mm S.E.M.) PPD and were considered by the clinician to be the most diseased sites of the quadrant. They were sampled with three fine paper points as described [13], except for using a curette instead of rubber cups to remove supragingival plaque beforehand. From healthy subjects marginal plaque was collected from four palatinal or lingual gingival surfaces using a sterile curette. Samples from a subject were pooled in 1 ml of sterile phosphate-buffered saline, immediately frozen at −20° or −80°C, and transported deep-frozen to the microbiology laboratory. There, samples were randomly coded, defrosted, vortexed for 30 s at the maximum setting, split into 200-μl aliquots, and restored at −80°C until further analysis. Analyses were performed blinded to the respective investigator.

2.3. Bacterial strains

P. gingivalis strains OMZ 925 (ATCC 33277), OMZ 409, OMZ 924 (6/26), OMZ 308 (W50), and OMZ 923 (HN-99) were used as a source for amplification of *fimA* alleles I to V, respectively. OMZ 923, 924 and 925 were provided by I. Nakagawa (Osaka, Japan), OMZ 308 by P. Marsh (Salisbury, UK), OMZ 409 is an own isolate from a deep periodontal pocket. All strains have been described previously [6,9,14]. They were cultured anaerobically in FUM medium [14,15]. *Escherichia coli* DH5α was used as host for plasmids and was grown aerobically at 37°C in LB medium. When required for maintenance of plasmids, ampicillin was added (100 μg ml^{-1}).

2.4. Preparation of DNA

Chromosomal DNA from overnight grown cultures of *P. gingivalis* was isolated according to Ausubel et al. [16]. Plaque DNA was extracted using the high pure PCR template preparation kit (Roche Applied Science, Rotkreuz, Switzerland) with the following modifications: after thawing and vortexing, 50-μl aliquots of each sample were added to 150 μl sterile DNase-free H_2O in Eppendorf Biopur microtubes (Eppendorf, Vaudaux, Switzerland) and heated for 10 min at 95°C. Samples were then sonicated for 15 min at 47 kHz at room temperature in a sonicator bath (Branson, model 2510, Soest, The Netherlands). Digestion with proteinase K and isolation of DNA were carried out following the manufacturer's instructions. Purified DNA samples were stored at −20°C.

2.5. Standards for quantitative PCR

Plasmids that served as standards for quantitative PCR were generated by PCR amplification from chromosomal DNA of *P. gingivalis*. PCR was carried out on a T-gradient thermocycler (Biometra, Göttingen, Germany). Reaction mixtures contained 1 μM of each primer, 0.5 mM dNTPs, 2 mM $MgSO_4$ and 1 U of Vent DNA polymerase England in $1\times$ Vent DNA polymerase reaction buffer. Cycling conditions were as follows: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, followed by final extension at 72°C for 7 min. The PCR products were polished with T4 DNA polymerase and phosphorylated with polynucleotide kinase. The DNA fragments were then cloned in pBluescript KS (Stratagene Europe, Amsterdam, The Netherlands) that had been digested with *Eco*RV and dephosphorylated with calf intestinal phosphatase. Sequencing of the inserts showed that the correct fragment had been cloned.

2.6. Quantitative PCR

Table 1 lists the primers used, the length and the melting

Table 1
P. gingivalis 16S rRNA-specific, *fimA*-specific and universal eubacterial DNA-specific primers used in the present study

Primer set	Sequence	Reference	Length of product (bp)	Melting point (°C)
P. gingivalis 16S rRNA forward	TGT AGA TGA CTG ATG GTG AAA ACC	[24]	197	87.5
Universal 16S rRNA reverse	ACG TCA TCC CCA CCT TCC TC	[24]		
fimA type I forward	CTG TGT GTT TAT GGC AAA CTT C	[6]	392	86
fimA type II forward	ACA ACT ATA CTT ATG ACA ATG G	[6]	257	83.5
fimA type III forward	ATT ACA CCT ACA CAG GTG AGG C	[6]	247	82.5
fimA type IV forward	CTA TTC AGG TGC TAT TAC CCA A	[6]	251	83.5
fimA type I to IV reverse	AAC CCC GCT CCC TGT ATT CCG A	[6]		
fimA type V forward	AAC AAC AGT CTC CTT GAC AGT G	[7]	462	84
fimA type V reverse	TAT TGG GGG TCG AAC GTT ACT GTC	[7]		
Universal 16S rRNA p8FPL	AGT TTG ATC CTG GCT CAG	[17]	834	85–89
Universal 16S rRNA p806R	GGA CTA CCA GGG TAT CTA AT	[17]		

point of the amplified DNA segments. Quantitative PCR was carried out on a LightCycler System (Roche Applied Science) using the FastStart DNA Master SYBR Green I kit (Roche Applied Science). PCR reactions were set up in glass capillaries in a total volume of 20 μl containing FastStart reaction mix and enzyme, 3.5 mM $MgCl_2$, 2 μl DNA and 1 μM of each primer. Temperature cycling profiles were as follows: 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. The detection of fluorescent products was monitored once every cycle selectively in the area around the melting point. After amplification, melting curve analysis was carried out in the range from 65 to 95°C to confirm that the DNA PCR products from plaque and reference plasmids had identical melting points. Selected PCR products were also assessed by electrophoresis in 1% agarose gels. In cases of very faint bands, the products were reamplified by PCR using Platinum Taq Polymerase (Invitrogen, Basel, Switzerland) and the following cycling conditions: 2 min preincubation at 95°C followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 7 min. Sequencing of selected *fimA* IV and *fimA* V PCR products was performed by Microsynth (Balgach, Switzerland) using one of the two primers that were employed for amplification. *fimA* type I PCR products were sequenced after cloning in the vector pBluescript KS.

Quantification of *P. gingivalis* copy numbers was done by comparison with standard curves, obtained with dilutions of 2.5 to 10^5 plasmids harboring a cloned copy of the template as described above. To exclude that compounds present in plaque DNA preparations inhibited PCR amplification, *P. gingivalis* 16S rRNA-negative extracts were spiked with 10^3 copies of the plasmid containing the rRNA template and retested. In addition, standard PCR with the broad-range eubacterial 16S rRNA primers p806R and p8FPL [17] was used to verify that sufficient amounts of eubacterial template DNA were present in *P. gingivalis*-negative extracts. Finally, to avoid the propensity of PCR assays to cause bias and artifacts when amplifying DNA from complex clinical or environmental samples at high PCR cycle numbers [18], the cycle number was limited to 40.

2.7. Immunofluorescence (IF)

Indirect IF with monoclonal antibody (mAb) 61BG1.3 [14] to enumerate *P. gingivalis* in plaque samples was performed exactly as described [19].

2.8. Statistical analyses

Data from 2×2 contingency tables were evaluated by Fisher's exact test. The null hypothesis that there was no difference between the colonization of *P. gingivalis*-positive samples with various *P. gingivalis* genotypes was assessed with the Friedman test. The rejection limit for the null hypothesis was set at 5%. In case of rejection, pairwise differences between genotypes were evaluated with the Wilcoxon signed rank test. All statistical analyses were done with the StatView 5.01 software (SAS Institute, Cary, NC, USA).

3. Results

P. gingivalis was detected in 71% of the periodontitis and 9% of the control samples (Table 2). With one exception (2%), presence or absence of the species was recorded identically by real-time PCR and IF (Table 2). For most positive samples real-time PCR amplification of *P. gingivalis*-specific 16S rRNA yielded cell estimates between 10^6 and 4×10^7 cells (Fig. 1A). The accuracy of these numbers was verified by indirect IF using a blinded protocol. Fig. 1B describes the excellent correlation of these data ($P<0.0001$, $r=0.964$). PCR and IF values differed by less than a factor of 3 with 12 of the 15 *P. gingivalis* positive samples. Repetitive sampling (3 weeks apart) of 11 Norwegian subjects yielded concurrent results (data not shown).

Distribution and quantity of *fimA* genotypes are sum-

Table 2
Presence or absence of *P. gingivalis* in relation to health status and detection assay

	Number of samples[a]		Number of samples[b]	
	Healthy subjects	Periodontitis patients	IF-pos	IF-neg
PCR-pos	3	12	14	1
PCR-neg	30	5	0	35

[a]Distribution of sample numbers in cells of the 2×2 contingency table indicates significantly different prevalence of *P. gingivalis* in healthy and diseased subjects (Fisher's exact test; $P < 0.0001$).
[b]Distribution of sample numbers in cells of the 2×2 contingency table demonstrates a highly significant relationship between these two principally different microbial detection assays (Fisher's exact test; $P < 0.0001$).

marized in Table 3. Each genotype was observed in at least two samples. With positive scores in 10 (67%) and 11 cases (73%), respectively, genotypes II and IV were most prevalent. More than two thirds of the *P. gingivalis*-positive samples (73%) harbored multiple *fimA* genotypes, whereas one sample was non-typable in spite of testing positive for *P. gingivalis* by both IF and 16S rRNA typing. Quantitatively, *fimA* genotype II predominated, exceeding the other genotypes in nine of the 10 samples that harbored genotype II. Genotype I was found in only two patients, but in both cases with high cell numbers. Genotype IV predominated in three samples, which notably were negative for the otherwise predominating genotype II. Genotypes III and V occurred in one third of the samples, but generally at very sparse densities. Overall, the *fimA* genotypes differed significantly ($P = 0.0016$; Friedman test) in their colonization of dental plaque, due primarily to the predominance of genotype II (Table 3). The identity of the PCR products was verified by melting curve analysis (Fig. 2A) and confirmed selectively by gel electrophoresis (Fig. 2B, Table 3) and DNA sequencing (Table 3).

4. Discussion

This report describes probably the first application of quantitative real-time PCR assays for the enumeration of *P. gingivalis fimA* genotypes in plaque. Quantitative measurements are of importance since likely not the mere presence but the abundance of the organism is of importance with respect to destructive periodontal disease. With complex clinical samples containing hundreds of bacterial species and frequently $>10^8$ bacteria per ml of sample solution, we used assay parameters that limited detection to 10 copies per PCR assay (<40 cycles, relative fluorescence threshold set to 0.1) to avoid possible interference from PCR-generated artifacts [18]. With this deliberate restriction the real-time PCR assay had a lower detection limit of 2×10^4 cells ml^{-1}, which is slightly higher than the corresponding limit of IF or DNA probe assays (approximately 5×10^3 cells ml^{-1}), but will be sufficient for most experimental or diagnostic applications. About half of the low signal PCR responses were verified by gel electrophoresis or DNA sequencing to confirm that the PCR products had indeed the required length or sequence for the respective *fimA* genotype. In all cases these analyses yielded the anticipated result (Fig. 2, Table 3). The results from the LightCycler PCR assay for the total cell number of *P. gingivalis* corresponded excellently with data from an IF test that measured the species' cell number with mAb 61BG1.3 that is directed to a conserved hemagglutinin epitope [20]. These findings demonstrate and confirm [21,22] the validity of quantitative real-time PCR for measuring of *P. gingivalis* in complex clinical samples. Melting curve analysis of real-time PCR amplification products

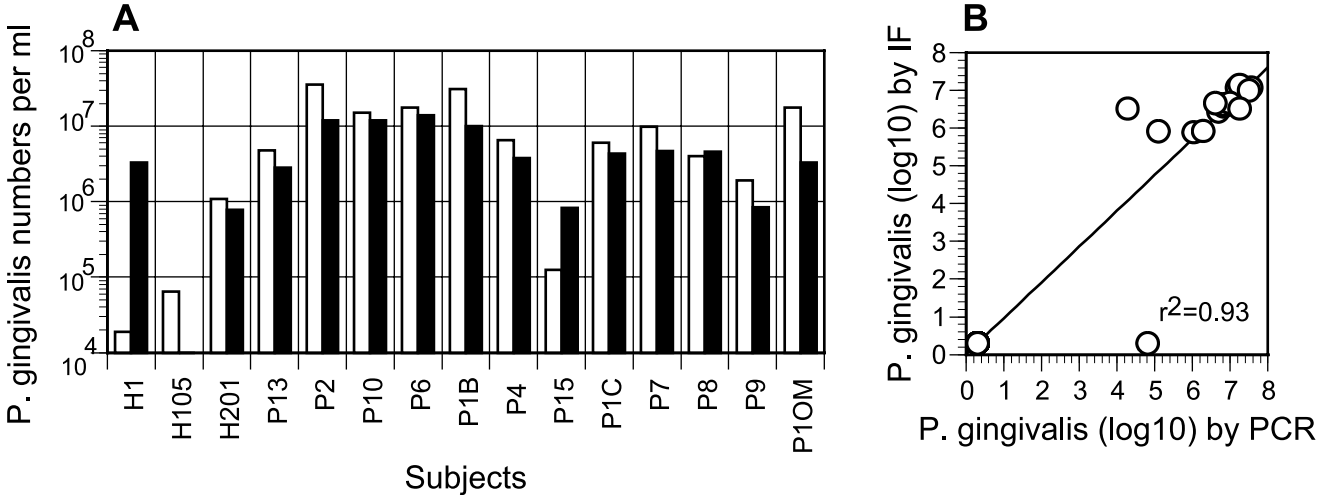

Fig. 1. *P. gingivalis* cell numbers detected by quantitative PCR and IF. A: Cell numbers detected in positive samples by PCR amplification of 16S rDNA (white bars) are compared to cell numbers counted using indirect IF with mAb 61BG1.3 (black bars). B: Correlation between results from quantitative PCR and IF analyses ($n = 50$). To enable log transformation, negative scores were replaced by a value of 2. The circle in the lower left corner corresponds to multiple data points.

Table 3
Differential distribution of multiple *fimA* genotypes in samples positive for *P. gingivalis*[a]

Sample[b]	*fimA* I[c,e]	*fimA* II[c,e]	*fimA* III[c,e]	*fimA* IV[c,e]	*fimA* V[c,e]	Sum of *fimA* I–V	16S rRNA[d,e]
H1	0	4.1	0	0.4	1.2[f]	5.7	0.2[d,e]
H105	0	25	0	0	0	25	0.6
H201	0	0	0	4.5[f]	0	4.5	11
P13	0	103	2.2	0.3[f]	0.3[f,g]	106	48
P2	0	882	1.8	0.2	0	884	359
P10	0	0	17	165	0	181	151
P6	0	97	0	1.2[f,g]	0.2[f,g]	98	177
P1B	0	315	0	0.4[f,g]	0	316	311
P4	0	146	2.5	0	0	149	66
P15	0	0	0	0	0	0	1.2
P1C	0	0	3.1	59	0.8[f,g]	63	61
P7	89[g]	221	0	0	0	310	98
P8	0	0	0	36	0	36	40
P9	0	48	0	7.4	0	56	19
P1OM	550[g]	5.0	0	1.5[f]	0.4[f]	557	177

[a]Pairwise comparison of scores for *fimA* II with those for *fimA* I, *fimA* III, *fimA* IV, and *fimA* V showed *P* values of 0.0593, 0.0076, 0.0962, and 0.0044, respectively (Wilcoxon test).
[b]'H' and 'P' in the code of a sample indicate that it had been collected from a healthy subject or a periodontitis patient, respectively.
[c]Number of cells ($\times 10^5$ ml^{-1} of sample) carrying the respective *fimA* allele.
[d]Number of cells ($\times 10^5$ ml^{-1} of sample) detected by assaying with 16S rRNA-specific primers.
[e]Data represent means of triplicate or, in a few cases, duplicate experiments.
[f]The identity of the PCR product was confirmed by gel electrophoresis.
[g]The identity of the PCR product was confirmed by DNA sequencing.

from plaque proved to be a highly valuable tool to assess quickly identity with reference sequences.

Investigating the abundance of *P. gingivalis fimA* genotypes in European Caucasians, a strikingly high percentage (73%) of samples with plaques from four sites were found colonized with two or more genotypes. Of interest was that the five genotypes seemed to differ markedly in their ability to proliferate to high (and potentially harmful) cell densities. Standing out in this respect was genotype II, which at a prevalence of 67% exceeded other *fimA* genotypes within the same sample on average by a factor of 184. Another interesting observation was that genotype IV, with a prevalence of 73%, reached high cell densities (in excess of 10^6 cells ml^{-1} of sample) only thrice and this only in the absence of the otherwise abundant genotype II. Genotypes I, III and V were clearly less prevalent; with 13% genotype I had the lowest prevalence but, if present, it reached comparatively high cell density. This pilot study has two major limitations, the small number of test persons and the assessment of pooled instead of single plaque samples. Due to the latter limitation co-existence of different *fimA* genotypes in the same ecological niche (site) could not be proved. Under conditions of proven co-colonization such findings could suggest differential control of *fimA* genotypes by host factors, by other biofilm members, or a competitive advantage of genotype II over genotype IV. Therefore, future studies devoted to this topic are of interest, but will have to assess individual sites in

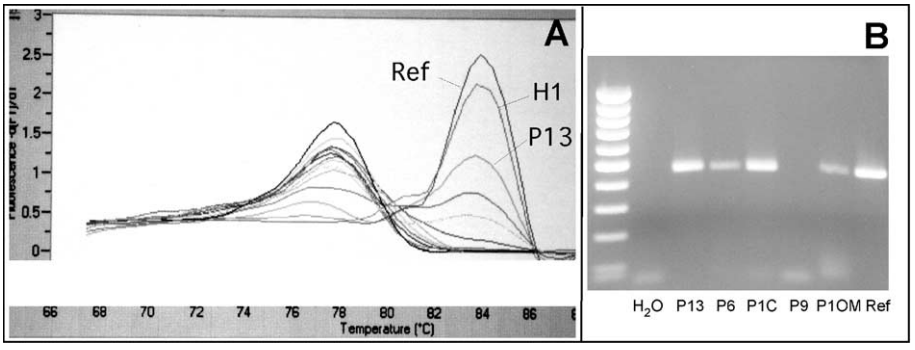

Fig. 2. Confirmation of identity of *P. gingivalis fimA* V PCR products. A: Representative melting curve analysis from real-time PCR. Each curve represents a different plaque sample. The *fimA* V amplification product of 462 bp has a melting point of approximately 84°C (right peak). To eliminate fluorescence emitted by primer–dimer complexes which melt at approximately 78°C (left peak), the detection of the *fimA* V product was monitored at every cycle near the melting point (83°C). Ref, standard curve obtained with a defined number of plasmids harboring a cloned copy of the *fimA* V PCR product. B: *fimA* V gene PCR products amplified from the indicated five plaque samples (lanes 3–7) and from the reference strain OMZ 923 (lane 8). The first lane contains the Gene Ruler™ 100-bp DNA size marker (MBI Fermentas, Vilnius, Lithuania), the second lane received water instead of a PCR product for amplification. All positive samples demonstrate a single product of the anticipated size, P9 was used as a negative control (Table 3).

order to better relate colonization by (a) certain genotype(s) to a particular ecological niche.

A comparison of our results with those of Amano et al. [8], who studied the presence or absence of the *fimA* genotypes in a large group of Japanese subjects originating from a limited area near Osaka, reveals both agreements and marked differences. First, the well-documented association of *P. gingivalis* with generalized chronic periodontitis [1] was confirmed once more. Second, the prevalence of the species in this study's subject groups was much lower than in the Japanese subject groups, in the periodontally healthy group the difference was four-fold (9.1% versus 36.3%). This cannot be explained by the younger mean age of our control subjects since the youngest quartile of Amano et al.'s control group still had a *P. gingivalis* prevalence of 35.5%. Third, with 73% against 27% the frequency of samples harboring multiple genotypes was much higher in the present study. Probably, this reflects different colonization patterns in subjects from different ethnicities with distinct cultural habits (e.g., diet), although it could also be related to differential assay sensitivity. The latter explanation cannot be excluded without a parallel testing of samples. However, we consider it unlikely since our PCR assay's lower detection limit was deliberately restricted. Fourth, we did not observe the striking association of *fimA* genotypes I and II with health and chronic periodontitis, respectively, that was reported for the Japanese subjects. In the present study genotype I was rare and not detected in periodontally healthy subjects. Unfortunately, the low prevalence of genotype I together with the relatively small sample number do not allow conclusions on the association of *fimA* genotypes I and II with periodontal health or disease in Europeans.

Besides genotype II fimbrillin expression, certain heteroduplex types of the ribosomal intergenic spacer region have been linked to human periodontitis [23]. Strains with close relationship to W83 – a reference strain for *fimA* genotype IV – were found to be significantly associated with chronic periodontitis, whereas strains that grouped with A7A1, a marker strain of *fimA* genotype II, showed no disease association because of their frequent presence at healthy sites [23]. Our results correspond with Griffen et al.'s observations [23] in two respects: the high frequency of samples containing multiple *P. gingivalis* strains and the dominating prevalence of the genotypes represented by A7A1 and W83.

In summary, the present study describes the application of a new quantitative real-time PCR assay for different fimbrial genotypes of *P. gingivalis* to a small number of samples from volunteers with or without chronic periodontitis. It shows that *P. gingivalis*-positive samples harbored mostly two or more *fimA* genotypes. Apart from differences in prevalence, the genotypes seemed to differ in their ability to reach high cell densities, possibly due to differential sensitivity to host defense mechanisms or to a competitive advantage of genotype II. To verify this hypothesis, further investigations with large numbers of site-specific samples are desirable. In view of the presumably very low prevalence of some *fimA* genotypes, the test groups (in particular the control group) will likely have to exceed 500 subjects – each with multiple test sites for investigation – to guarantee that every genotype is detected with statistically sufficient frequency.

Acknowledgements

We thank I. Nakagawa (Osaka University, Japan) for the gift of reference strains representing the *P. gingivalis fimA* genotypes. The excellent technical assistance from Verena Osterwalder, Martin Gander and Yvonne Helweg is gratefully acknowledged. This investigation was supported by the Universities of Zürich and Bergen.

References

[1] Slots, J. and Listgarten, M.A. (1988) *Bacteroides gingivalis, Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. J. Clin. Periodontol. 15, 85–93.

[2] Holt, S.C., Kesavalu, L., Walker, S. and Genco, C.A. (1999) Virulence factors of *Porphyromonas gingivalis*. Periodontol. 2000 20, 168–238.

[3] Yoshimura, F., Sugano, T., Kawanami, M., Kato, H. and Suzuki, T. (1987) Detection of specific antibodies against fimbriae and membrane proteins from the oral anaerobe *Bacteroides gingivalis* in patients with periodontal diseases. Microbiol. Immunol. 31, 935–941.

[4] Dickinson, D.P., Kubiniec, M.A., Yoshimura, F. and Genco, R.J. (1988) Molecular cloning and sequencing of the gene encoding the fimbrial subunit protein of *Bacteroides gingivalis*. J. Bacteriol. 170, 1658–1665.

[5] Loos, B.G. and Dyer, D.W. (1992) Restriction fragment length polymorphism analysis of the fimbrillin locus, *fimA*, of *Porphyromonas gingivalis*. J. Dent. Res. 71, 1173–1181.

[6] Amano, A., Nakagawa, I., Kataoka, K., Morisaki, I. and Hamada, S. (1999) Distribution of *Porphyromonas gingivalis* strains with *fimA* genotypes in periodontitis patients. J. Clin. Microbiol. 37, 1426–1430.

[7] Nakagawa, I., Amano, A., Kimura, R.K., Nakamura, T., Kawabata, S. and Hamada, S. (2000) Distribution and molecular characterization of *Porphyromonas gingivalis* carrying a new type of *fimA* gene. J. Clin. Microbiol. 38, 1909–1914.

[8] Amano, A., Kuboniwa, M., Nakagawa, I., Akiyama, S., Morisaki, I. and Hamada, S. (2000) Prevalence of specific genotypes of *Porphyromonas gingivalis fimA* and periodontal health status. J. Dent. Res. 79, 1664–1668.

[9] Frandsen, E.V.G., Poulsen, K., Curtis, M.A. and Kilian, M. (2001) Evidence of recombination in *Porphyromonas gingivalis* and random distribution of putative virulence markers. Infect. Immun. 69, 4479–4485.

[10] Kilian, M. (1998) Clonal basis of bacterial virulence. In: Oral Biology at the Turn of the Century. Misconceptions, Truths, Challenges and Prospects (Guggenheim, B. and Shapiro, S., Eds.), pp. 131–142. Karger, Basel.

[11] Loos, B.G., Dyer, D.W., Whittam, T.S. and Selander, R.K. (1993) Genetic structure of populations of *Porphyromonas gingivalis* associated with periodontitis and other oral infections. Infect. Immun. 61, 204–212.

[12] Glavind, L. and Löe, H. (1967) Errors in the clinical assessment of periodontal destruction. J. Periodont. Res. 2, 180–184.
[13] Gmür, R., Strub, J.R. and Guggenheim, B. (1989) Prevalence of *Bacteroides forsythus* and *Bacteroides gingivalis* in subgingival plaque of prosthodontically treated patients on short recall. J. Periodont. Res. 24, 113–120.
[14] Gmür, R., Werner-Felmayer, G. and Guggenheim, B. (1988) Production and characterization of monoclonal antibodies specific for *Bacteroides gingivalis*. Oral Microbiol. Immunol. 3, 181–186.
[15] Gmür, R. and Guggenheim, B. (1983) Antigenic heterogeneity of *Bacteroides intermedius* as recognized by monoclonal antibodies. Infect. Immun. 42, 459–470.
[16] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1992) Short Protocols in Molecular Biology. John Wiley and Sons, New York.
[17] Relman, D.A., Schmidt, T.M., MacDermott, R.P. and Falkow, S. (1992) Identification of the uncultured bacillus of Whipple's disease. New Engl. J. Med. 327, 293–301.
[18] Qiu, X., Wu, L., Huang, H., McDonel, P.E., Palumbo, A.V., Tiedje, J.M. and Zhou, J. (2001) Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl. Environ. Microbiol. 67, 880–887.
[19] Gmür, R. and Thurnheer, T. (2002) Direct quantitative differentiation between *Prevotella intermedia* and *Prevotella nigrescens* in clinical samples. Microbiology 148, 1379–1387.
[20] Curtis, M.A., Aduse-Opoku, J. and Rangarajan, M. (2001) Cysteine proteases of *Porphyromonas gingivalis*. Crit. Rev. Oral Biol. Med. 12, 192–216.
[21] Lyons, S.R., Griffen, A.L. and Leys, E.J. (2000) Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. J. Clin. Microbiol. 38, 2362–2365.
[22] Martin, F.E., Nadkarni, M.A., Jacques, N.A. and Hunter, N. (2002) Quantitative microbiological study of human carious dentine by culture and real-time PCR: Association of anaerobes with histopathological changes in chronic pulpitis. J. Clin. Microbiol. 40, 1698–1704.
[23] Griffen, A.L., Lyons, S.R., Becker, M.R., Moeschberger, M.L. and Leys, E.J. (1999) *Porphyromonas gingivalis* strain variability and periodontitis. J. Clin. Microbiol. 37, 4028–4033.
[24] Tran, S.D. and Rudney, J.D. (1999) Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, and *Porphyromonas gingivalis*. J. Clin. Microbiol. 37, 3504–3508.