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Genetic variability of *Porphyromonas gingivalis* through two virulence factors isolated from patients with and without periodontitis

Variabilità genetica di Porphyromonas gingivalis isolato in pazienti con e senza parodontite, analizzata attraverso due fattori di virulenza

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Summary

The pathogenicity of P.g. is expressed by a lot of virulence factors. The aim of this cross-sectional study is to analyze genetic variability and prevalence of the genes codifying for gingipains and fimbriae of P.g. A wide variability in the prevalence and distribution of those genes is shown. A possible association was found between one type of P.g. fimbriae and periodontitis patients.

Riassunto

La patogenicità di *P.g.* è determinata dall'espressione di svariati fattori di virulenza. L'obiettivo di questo studio trasversale è quello di analizzare la variabilità genetica e la prevalenza dei geni codificanti per le gingipaine e le fimbriae di *P.g.* La prevalenza e la distribuzione di questi geni risultò essere ampiamente variabile. Si incontrò una possibile associazione tra i pazienti con parodontite e *P.g.* con un determinato tipo di fimbria.

Introduction

Porphyromonas gingivalis (P.g.) is considered one of the most pathogenic bacteria in the aetiology of periodontal diseases. This high pathogenicity has been attributed to the expression by the bacteria of a wide range of virulence factors, such as ex-

ternal membrane proteins (lipoproteins, carriers proteins), lipopolysaccharides, proteases (tripsyn-protease, collagenase, aminopeptidase, gingipains), lipolytic enzymes, capsular polysaccharides (K-Antigens) and fimbriae . Among these virulence factors, the presence of fimbriae has received increased attention due to its clear association with the microbe pathogenicity (1). Six type of fimbriae have been characterized as type I to type V *fimA,* together with type Ib *fimA* (2). From the reviewed studies , it can be derived that type II *fimA* genotype is the most prevalent in periodontitis patients, followed by type IV, Ib, and type V. These associations point out towards a possible correlation between type II *fimA* and increased pathogenicity. Other virulence factors of this bacterium are involved in the process of periodontal tissue destruction. The most important proteolytic activity of P. gingivalis is played by a family of major cysteine proteinases called, gingipain (Arg- gingipain (Rgp) and Lys-gingipain (Kgp). Three types (type A, B and C) of the Arg-specific cysteine proteinase genes (*rgpA*) and two (type I and II) of the Lys-specific cysteine proteinase genes (*kgp*) were identified (3,4). It has been suggested that Arg and/or Lys gingipains may play a variety of physiological roles in P. gingivalis, including controlling the expression and/or processing of virulence factors (5).

The aim of this study was to study the genetic variability and the prevalence of the genes codifying for two virulence factors of *P. gingivalis:* gingipains and fimbriae from species isolated from patients with and without periodontitis.

Material and Methods

Subjects: consecutive subjects were sampled from both healthy volunteers and periodontitis patients referred to the Postgraduate Clinic of Periodontology at the Faculty of Odontology from the University Complutense of Madrid.

Periodontal conditions and examination. Chronic periodontitis and aggressive periodontitis were defined according to the criteria described by Armitage (1999) (6) plus the presence of more than 5 sites with probing pocket depths ≥ 6 mm, at least one per quadrant. A diagnosis of gingivitis was made if the patient showed less than 5 sites with a pocket depth ≥ 6 mm, and presented more than 25% of the sites with bleeding on probing. If the bleeding index was lower than 20%, a healthy status was ascribed.

Microbiological Sampling. Four sites, one in each quadrant, were selected based on the patient's periodontal status, selecting the sites with deeper pockets that bled upon probing. The paper points were transferred to a vial containing 1.5 mL of Reduced Transport Fluid (RTF), and pooled with all the other paper points taken from the other quadrants. The vial was sent to the laboratory and processed within 24 hours.

Genomic DNA extraction and purification: whole DNA extraction was carried out by phenol-chloroform extraction. Genomic DNA were analyzed by means of agarose gel electrophoresis in 1X TAE buffer and visualized by staining with ethidium bromide.

fimA, kgp and rgp typing in P. gingivalis isolates: genotype determination was achieved by PCR and PCR-RFLP. The selected primers were previously described. PCR assays were performed in a thermal cycler (TC-412, TECHNE). The PCR products were subjected to electrophoresis in 1-5% agarose gel 1X Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and photographed under UV illumination.

Data analysis: prevalence data is presented in percentages, first in relation to presence of different fimbriae types by patients and then in relation to strains. Differences in presence/absence according to periodontal status were analyzed in contingency tables and compared by means of the Fisher´s Exact test. Odds ratio (OR) and 95% Confidence Intervals (CI) were also calculated, using the Woolf's approximation. The level of significance was established at 95%. For appropriate data calculation no presence data was adjusted to 0.5 in order to avoid zero values. Descriptive analysis of frequency detection has been reported for data corresponding to *kgp* and *rgp* genes analysis.

Results

P.g. strains were isolated from 39 patients with and without periodontitis for the analysis of *fimA genes. P.g.* isolates from 46 and 47 patients, were analyzed for the determination of genetic variability of *rgpA* and *Kgp* genes, respectively. In addition, six capsular serotypes of P. gingivalis, (K1) W50, (K2) Hg184, (K3) HG1025, (K4) ATCC 49417, (K5) HG1690, (K6) HG1691 and one non-capsular strain, (K-) ATCC 33277, were used for PCR amplification (Table 1).

fimA, kgp and rgp genes identification in known K-serotypes of P.g.

Table 1. fimA types of capsular serotypeable strains

Genotype	Capsular serotype	***fimA* type**	***rgpA* type**	***Kgp* type**
ATCC 33277	(K-)	*fimA* I	*rgpA* D	*Kgp* I
W50	(K1)	*fimA* IV	*rgpA* D	*Kgp* II
HG184	(K2)	*fimA* II	*rgpA* D	*Kgp* I
(A7A1-28) HG1025	(K3)	*fimA* II	*Not identified*	*Kgp* II
ATCC49417	(K4)	*fimA* III	*rgpA* A	*Kgp* I
HG1690	(K5)	*fimA* II	*rgpA* A	*Kgp* I
HG1691	(K6)	*fimA* Ib	*rgpA* A	*Kgp* II

Table 1 shows the prevalence of the *fimA* genes in seven laboratory strains of *P. gingivalis.*

Frequency of detection and distribution of fimA genes

From all the studied subjects, 31 (79.5%) harboured only one *P.g.* fimbria type, while in the remaining 8 subjects two different types were detected. The detection of two fimbriae types was more frequent in non-periodontitis patients (73.3%,

OR=0.55 (CI: 0.11-2.64)) than in periodontitis patients (83.3%, OR=1.82 (CI: 0.38-8.73)), although these differences were not statistically significant (p=0.686).

Among the different types of fimbriae, type II was the most frequent *fimA* gene (14 subjects harboured it as the only fimbriae type) and also the most frequent type in periodontitis patients (45.8%). The second was *P.g.* fimbria type I, isolated in 6 subjects (15.4%), and being most frequently isolated in non-periodontitis subjects (4 subjects, 26.7%). Other types of fimbriae or combinations were less frequent, and no differences were detected between non-periodontitis and periodontitis patients. When OR were calculated, type II was associated with periodontitis (OR=3.38, CI: 0.76-15.15), and type I showed the opposite tendency (OR=0.25, CI: 0.04-1.58). None of the results from the contingency tables reached the level of statistical significance.

Table 2 shows the distribution of *P.g.* fimbriae types within the isolates (3-5 isolates were subcultured from each patient). Again, the most frequent type was type II, with 74 isolates (46.8%). Type I, IV and Ib were found in 23-28 strains, while types III and V were less frequent. In periodontitis patients, *P.g.* fimbriae type II was found in 55.3% of the isolates, while types IV and Ib in less than 20%. In non-periodontitis subjects, its prevalence was distributed more evenly, with around 30% corresponding to types I and II, and between 15-20% to types IV and Ib. The

Table 2. Frequency of detection of different type of fimbriae of isolates from patients with different periodontal conditions

							Odds ratio (95% CI) and p values		
FIMBRIAE	All isolates (n)	All isolates (%)	Non periodontitis (n)	Non periodontitis (%)	Periodontitis (n)	Periodontitis (%)	OR	95% CI	Fisher´s Exact test
I	26	16.5%	18	28.1%	8	8.5%	0.2377	0.09600 to 0.5887	0.0018
II	74	46.8%	22	34.4%	52	55.3%	2.3640	1.225 to 4.559	0.0146
III	2	1.3%	2	3.1%	0	0.0%	0.1323	0.006240 to 2.804	0.1625
IV	28	17.7%	10	15.6%	18	19.1%	1.2790	0.5476 to 2.987	0.6731
V	5	3.2%	0	0.0%	5	5.3%	7.9270	0.4304 to 146.02	0.0812
Ib	23	14.6%	12	18.8%	11	11.7%	0.5743	0.2361 to 1.397	0.2540
TOTAL	158	100.0%	64	100.0%	94	100.0%			

analysis from the contingency tables demonstrated a significant association between type II with periodontitis patients (p=0.015, OR=2.36 (CI: 1.22-4.56) and a trend towards significance for type V (p=0.081, OR=7.93 (CI: 0.43-146.02). Conversely, an inverse association was found for type I (p=0.02, OR=0.24 (CI: 0.09-0.59).

Frequency of detection and distribution of rgpA and Kgp genes

It was possible to discriminate the presence of type I and II *kgp* genotype and the presence of four types of *rgpA* gene (A, B, C and D). *rgpA* and *Kgp* genes were detected in all *P.g.* isolates from patients with and without periodontitis. *rgpA* gene type A was the most frequent detected among all isolates while other types of this genes were generally detected with almost the same frequency. In the same way

type A of *rgpA* was the most frequent detected in both, periodontitis and non-periodontitis patients followed by type C and B. In contrast *rgpA* type D was detected only in 1 non periodontitis patient (Table 3). Combination of two types of rgpA genes in the same patient seems to be more frequent in periodontitis patients. *Kgp* analysis showed an even more heterogeneous detection between isolates coming from patients with and without periodontitis. Most of the patients have been found harbouring one type of *Kgp* gene. A slight higher prevalence of *Kgp* II gene was found in non periodontitis patients, in contras *Kgp* type I was slightly more prevalent in non-periodontitis patients. In general *Kgp* type I was the most frequent detected in our samples (Table 4).

Table 3. Prevalence of different types of rgpA genes or combination of those genes in patients with different periodontal conditions

rgpA type	All subjets (n)	All subjects (%)	Non periodontitis (n)	Non periodontitis (%)	Periodontitis (n)	Periodontitis (%)
A	25	54.3%	8	53.3%	17	54.8%
B	6	13.0%	2	13.3%	4	12.9%
C	9	19.6%	3	20.0%	6	19.4%
D	1	2.2%	1	6.7%	0	0.0%
A/B	1	2.2%	0	0.0%	1	3.2%
A/C	3	6.5%	0	0.0%	3	9.7%
A/D	1	2.2%	1	6.7%	0	0.0%
TOTAL	46	100.0%	15	100.0%	31	100.0%
Only one *rgpA* type	41	89.1%	14	93.3%	27	87.1%
Two *rgpA* types	5	10.9%	1	6.7%	4	12.9%

Table 4. Prevalence of different types of Kgp genes or combination of those genes in patients with different periodontal conditions

Kgp type	All subjets (n)	All subjects (%)	Non periodontitis (n)	Non periodontitis (%)	Periodontitis (n)	Periodontitis (%)
1	26	55.3%	10	66.7%	16	50.0%
2	14	29.8%	3	20.0%	11	34.4%
1/2	7	14.9%	2	13.3%	5	15.6%
TOTAL	47	100.0%	15	100.0%	32	100.0%
Only one *Kgp* type	40	85.1%	13	86.7%	27	84.4%
Two *Kgp* types	7	14.9%	2	13.3%	5	15.6%

Discussion

The results from this study confirm previous reports that genetic variability among isolates of *P. gingivalis* from patients with different periodontal conditions exists. We found that the distribution of *P.g.* fimbriae types in isolates from non-periodontitis and periodontitis subjects is different. In periodontitis subjects, a higher frequency

of detection of type II *fim*A gene was detected, while the prevalence of other types was low. In non-periodontitis subjects, the distribution in fimbriae types was more homogeneous with a relatively high prevalence of type I *fim*A gene. High prevalence of types II, IV and Ib *fimA* genes were also demonstrated in patients with periodontitis, while higher prevalence of type I *fimA* were associated to subjects without periodontitis. Those results agree with previous published studies, where similar prevalence of *fimA* genes has been shown for subjects coming from different geographical areas. In terms of the *rgpA* and kgp genotypes we found that type A of *rgpA* gene is the most frequent detected in our samples followed by type C and B, while *kgp* type I was the most frequent detected in both periodontitis and non-periodontitis patients. Other studies reported that kgp and rgpA genotypes seem to have a similar frequency in subjects throughout different geographical regions (e.g. Japan, Germany, the UK, Sweden and Australia). In contrast with other studies we detected more than one type of *rgpA* and more than one type of *kgp* in isolates coming from the same patients. Moreover we found four types of *rgpA* genes that could results in a new unknown genotype.

This study, therefore, supports the hypothesis that the increased pathogenicity of *P.g.* is conferred by the presence specific *fimA* genotypes. This fact has also been supported by multiple in vitro studies demonstrating an increased adhesion and invasion capability to epithelial cells by certain *fimA* genotypes of *P.g*. Chen T. et al. (7) reported that specific *fimA* II clone (HW24D-1) of *P.g.* elicited an enhancement in the adhesion mechanism. Nevertheless, it has been also been demonstrated that adhesion of *P.g.* is possible in absence of fimbriae (7) and that expression of *fimA* II in *P.g.* clones is not sufficient to demonstrate invasiveness (8). The fact that certain types of fimbriae mediate in the capability of adhesion and invasion of *P.g.* (9) does not probably mean that fimbriae are the only factor implicated in these mechanisms. Other virulent factors on their cellular surface have also been implicated in bacterial-host interactions. Chen et al. have also shown that gingipain adhesins mediate both *P.g.* adherence to epithelial cells and through its proteolytic activity in its invasiveness. Gingipain catalytic activity (especially that of Arg-gingipains) may also modulate *P.g.* adhesion (10).

All these multiple *in vitro* studies clearly suggest that the pathogenicity of *P.g.* may be the result of complex interactions between different virulence factors. Moreover, expression of these virulence factors may be related to other factors, such as the bacterial growing conditions, the presence of available nutrients, favourable biofilm structures and bacterial compositions.

Conclusions

There is a clear genetic variability among isolates of *P. gingivalis* from patients with different periodontal conditions. In this study specific genotypes were found with different prevalence in the two groups, showing a possible association with their

specific periodontal health status. In periodontitis subjects, a higher frequency of detection of type II *fim*A gene was demonstrated, while the prevalence of other types was low. In non-periodontitis subjects, a more even distribution in fimbriae types was seen with a relatively high prevalence of type I *fim*A gene. *rgpA* and *Kgp* genotypes were detected from all isolates without remarkable differences between the two groups.

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