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Distribution of Genotypes of *Porphyromonas gingivalis* in Type 2 Diabetic Patients with Periodontitis in Mexico

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Abstract

Objective: To determine and compare the distribution of *Porphyromonas gingivalis fimA* genotypes in type 2 diabetes mellitus (T2DM) patients affected by periodontitis, using non-diabetic subjects with and without periodontitis as control groups.

Material and Methods: This study involved 75 subjects divided into three groups of 25 subjects each: Group 1 (non-T2DM without periodontitis), Group 2 (non-T2DM with periodontitis) and Group 3 (T2DM with periodontitis). The outcome variable was periodontitis, and explanatory variables were age, sex, T2DM and specific *P. gingivalis fimA* genotypes.

Results: In non-T2DM subjects with healthy periodontal tissues, type I *fimA* was the most frequently detected individually (40%) or in combinations (40%). In non-T2DM subjects with periodontitis, the most frequently detected type was Ib individually (20%) or in combinations (36%). In T2DM patients with periodontitis, the most frequently detected types were types I (20%) and III (20%), but there was no statistical difference ($p > 0.05$) with non-T2DM periodontitis subjects.

Conclusions: Type I genotype was more frequently detected in periodontally healthy sites from non-T2DM subjects than in periodontitis sites from either subjects with or without T2DM. However, in sites affected by periodontitis from T2DM subjects the predominating types were I and III, which are less virulent strains of *P. gingivalis*.

Key words: diabetes; *fimA*; genotype; *P. gingivalis*

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Periodontitis, one of the most common infectious diseases seen in humans, is a chronic inflammatory disease of the soft tissues supporting the teeth, which, in severe cases, leads to tooth loss (Williams & Offenbacher 2000). *Porphyromonas gingivalis*, a Gram-negative black-pigmented strictly anaerobic bacterium, has been implicated as a major aetiological agent in the onset and progression of chronic destructive periodontitis (Slots & Ting 1999). It should be noted that the presence of periodontal pathogens is necessary, although not sufficient for disease development, as the role of host inflammatory response appears to be the critical determinant for susceptibility and severity, especially in systemically compromised individuals such as diabetes mellitus (DM) patients (Genco 1996, Williams & Offenbacher 2000). In Mexico, in less than four decades, type 2 DM (T2DM) has become a major health problem, being a principal cause of death in women and the second among men since the year 2000. It is the primary cause of premature retirement, blindness and kidney failure; by the year 2025, it is expected that close to 11.7 million Mexicans will be diagnosed with DM (Rull et al. 2005). It has been reported that Mexican adolescents affected by DM with imbalance of glucose showed an association with periodontal disease (Patiño-Marin et al. 2002).

Accumulated evidence shows a high incidence and severity of periodontal disease in T2DM patients as compared with non-DM controls (Taylor et al. 1998, Taylor 2001). Severe periodontitis often coexists with diabetes and is considered to be the sixth complication of the disease (Löe 1993), as both type 1 and T2DM patients show a three- to four-fold increased risk of periodontitis (Taylor et al. 1998). Several studies indicated poorer periodontal health in

T2DM patients, who were 2.8 times as likely to have periodontitis and 4.2 times more likely to have alveolar bone progression (Taylor et al. 1998, Promsudthi et al. 2005). It is also suggested that successful management of periodontal infection may lead to control of glucose metabolism (Pickup & Crook 1998, Kiran et al. 2005). The increased risk for periodontal disease cannot be explained on the basis of age, gender or hygiene; besides, the glycaemic control of DM is a primary contribution in the prevalence and severity of periodontal disease (Taylor 2001, Promsudthi et al. 2005). Several mechanisms have been suggested for increased susceptibility to infection in DM including impaired acquired immunity due to insufficient chemotactic, phagocytic and bactericidal function as well as altered innate immune response by the host (Joshi et al. 1999). DM patients are susceptible to all kinds of infections; however, they seem to be particularly vulnerable to Gram-negative bacteria, which may be involved in urinary tract, soft-tissue and periodontal tissue infections (Patterson & Andriole 1997, Holt et al. 1999).

The pathogenicity of *P. gingivalis* has been attributed to several virulence factors, which include lipopolysaccharide, fimbriae, haemagglutinin, haemolysin, and extracellular proteinases (Lamont & Jenkinson 1998, Amano 2003, Imamura 2003, Nakayama 2003). Fimbriae are hair-like appendages considered to be critical virulence factors, which mediate bacterial interactions with and invasion of host tissues (Amano 2003). Fimbriae are involved in most adherence properties displayed by *P. gingivalis*, i.e., the interactions with salivary molecules, oral epithelial cells and other oral bacteria (Amano et al. 2004). These fimbriae have been classified into six genotypes (I–V, and Ib), based on the diversity of the *fimA* (a subunit protein of fimbriae) genes encoding FimA (Amano et al. 2000, Nakagawa et al. 2002). Studies have shown that clones with type II *fimA* have a significantly greater virulence *in vitro* and *in vivo* as compared with other genotypes, while types I and III are considered less virulent (Amano et al. 2000, 2004, Nakagawa et al. 2002, Nakano et al. 2004, Miura et al. 2005).

There are several studies that have detected and quantified subgingival periodontal pathogens in periodontally healthy tissues or diseased sites in systemically healthy patients, using the polymerase chain reaction (PCR) method (Riggio et al. 1996, Amano et al. 2000). However, there are no reports that have investigated *P. gingivalis fimA* genotypes in a population with a high prevalence of DM such as the Mexican population. The aim of the present study was to determine and compare the distribution of *fimA* genotypes of *P. gingivalis* in T2DM patients affected by periodontitis, using non-T2DM subjects with and without periodontitis as control groups.

Material and Methods

Patients

The Ethics Committee of the Master's Degree Program in Advanced General Dentistry at San Luis Potosi University, Mexico, approved this study. This case–control prospective study involved 75 non-smoking subjects divided into three groups of 25 subjects each: Group 1 (non-T2DM subjects without chronic periodontitis: eight males, 17 females; age 30–62 years), Group 2 (non-T2DM subjects with chronic periodontitis: five males, 20 females; age 30–70 years) and Group 3 (T2DM patients with chronic periodontitis: 13 males, 12 females; age 37–68 years). T2DM diagnostic criteria used were according to the American Diabetes Association (Shaw et al. 2000). Attendant risks and benefits were explained, and written consent was obtained from all patients. A non-probabilistic consecutive sampling was performed from January 2004, through August 2005. Patients were recruited at San Luis Potosi University and at Hidalgo State University, Mexico. For Group 1 (G1), Group 2 (G2) and Group 3 (G3), 66, 36 and 36 subjects, respectively, were recruited and 25 subjects who harboured *P. gingivalis* were included in each group. The exclusion criteria were as follows: all subjects who received professional cleaning, periodontal surgery and antibiotic medication within 3 months before the study. All variables included were blind-analysed in both institutions.

Periodontal examination and clinical specimens

Periodontal disease was determined by measuring the level of attachment loss (LAL) and probing pocket depth (PPD) at all teeth including the sampled tooth. Clinical diagnosis of periodontitis was given for LAL and PPD with more than 5 mm in at least 10 sites (Borrell & Papapanou 2005). Supragingival plaque was gently removed, and subgingival plaque samples from the disto-lingual surface of the left mandibular lateral incisor were collected from all patients, with sterile Gracey curettes. Samples were placed in Eppendorf sterile tubes containing 1 ml of phosphate-buffered saline (pH 7.4) on ice; all samples were maintained at -40°C until experiments were conducted. The specimens were vortex mixed and centrifuged at $12{,}000 \times g$ for 1 min. to pellet the bacterial cells. The bacterial genomic DNA was isolated with a DNA isolation kit according to the manufacturer's instructions (Puregene; Gentra Systems, Minneapolis, MN, USA), and the isolated DNA was dissolved in 100 μl of TE (10 mM Tris HCl [pH 8.0] and 1 mM EDTA) buffer.

Bacterial strains

P. gingivalis strains ATCC 33277 (type I *fimA*), HW24D1 (type II *fimA*), 6/26 (type III *fimA*), HG564 (type IV *fimA*), OMZ314 (type V *fimA*) and HG1691 (type Ib *fimA*) were provided by the Department of Oral Frontier Biology, Osaka University, Japan. These organisms were anaerobically grown in GAM broth (Nissui Co Ltd., Tokyo, Japan) as described previously (Nakagawa et al. 2002).

PCR primers and amplification

PCR was performed to detect *P.gingivalis* using specific primers for 16S rRNA, and then to identify its genotypes, specific primers for *fimA* were used, as described previously (Amano et al. 2000, Nakagawa et al. 2002). Briefly, the PCR assay was performed with a total volume of 25 μl consisting of PCR beads (Ready-To-Go; Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.8 μM each primer and 2–5 μl of the template DNA solution (20–60 μg/ml). The PCR reaction was performed in a thermal cycler (iCycler, BIO-RAD laboratories, Hercules, CA, USA) with the following cycling parameters: an initial denaturation at 95°C for 5 min., 40 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. The PCR products were analyzed by electro-

phoresis in a 2% agarose gel-Tris-acetate-EDTA buffer, and a 100-bp DNA ladder marker (New England Biolabs, Beverly, MA, USA) was used as a reference for molecular size (Amano et al. 2000). The gel was stained with 0.5 μg of ethidium bromide/ml and photographed under ultraviolet (UV) illumination (Chemi Doc, BIO-RAD laboratories, Hercules, CA, USA).

Sequencing

Five samples from G3 patients were positive for *P. gingivalis* 16S rRNA but negative for *fimA* type-specific primers. Their PCR products amplified with primers for *P. gingivalis* 16S rRNA were cloned in the pGEM T-Easy vector (Promega, Madison, WI, USA) and sent for sequencing at the CINVESTAV-IPN Research Institute in Irapuato, Mexico.

Statistical analysis

Two examiners were calibrated in all variables for the diagnosis of periodontal disease with an expert periodontist through an intra-class correlation coefficient. All data are expressed as mean $\pm$ standard deviation; JMP programme version 5.1 and Stat View (SAS Institute, USA) were used for statistical analysis, and statistical significance was set at $p<0.05$. Shapiro–Wilks, Levene and Brown Forsythe tests were used to test the normality of distribution. The Mann–Whitney *U* test was used to compare continuous variables; Fisher's exact and χ^2 of Mantel–Haenszel tests were used to compare categorical variables.

Results

Periodontal disease

The intra-class correlation coefficient between two examiners was 0.87. The prevalence of positive detection among the populations initially screened for *P. gingivalis* by means of the 16S rRNA gene was as follows: G1 38%, G2 70% and G3 70%. The means in G2 [PPD (5.24 ± 1.2) and LAL (6.40 ± 1.7)] and G3 [PPD (5.25 ± 1.15) and LAL (6.27 ± 2.07)] were significantly greater than those in G1 [PPD (1.91 ± 0.27) and LAL (1.05 ± 0.23)], and all variables showed a significant statistical difference ($p<0.05$) (Table 1). The periodontal condition of subjects included in each group is summarized in Table 1. There was no statistical difference in age ($p>0.05$, Mann–Whitney *U* test), levels of PPD and LAL between the groups affected by periodontal disease ($p>0.05$, χ^2 test). On comparing the periodontitis groups (G2 and G3) with the control group (G1), there was a statistically significant difference in periodontal condition (PPD and LAL, $p=0.001$).

Phylogenetic relationship between *fimA*-untypable clones and type I *fimA* clone

All *fimA*-untypable samples (C:19d, C:47a, C:31g, C:44g and C:43b) were from G3, which were not amplified with the *fimA* type-specific primers but produced a 197-bp PCR product with the 16S rRNA primers, which were sequenced. The 16S rRNA sequence of *P. gingivalis* clone with type I *fimA* (Accession Numbers: AF414809, AF414820 and L164492) was taken from the GenBank. The sequence of AF118634 (type III *fimA* clone) was used as an outgroup control. In addition, a 16S rRNA sequence from *Porphyromonas* endodontalis was used (L16491) as a reference in the phylogenetic tree. The analysis based in nucleotide alignment revealed that the *P. gingivalis* clones contained in these samples were closely related to the type I *fimA* clone, which suggested that untypable samples belong to *fimA* type I of *P. gingivalis* (Fig. 1).

Table 1. Periodontal condition of the subjects

	Group 1	Group 2	Group 3
PPD	1.91 ± 0.27	5.24 ± 1.2	5.25 ± 1.15
LAL	1.05 ± 0.23	6.40 ± 1.7	6.27 ± 2.07
AGE	42.7 ± 8.6	51.4 ± 10.3	49.1 ± 10.1
	G1 *versus* G2	G1 *versus* G3	G2 *versus* G3
PPD	p = 0.001	p = 0.001	p = 0.8538
LAL	p = 0.001	p = 0.001	p = 0.8843

PPD, probing pocket depth; LAL, level attachment loss. All data are expressed as mean $\pm$ standard deviation. Age: There was not a statistically significant difference between groups affected by periodontal disease ($p>0.05$) for all age values among groups. All *p*-values were from χ^2 test.

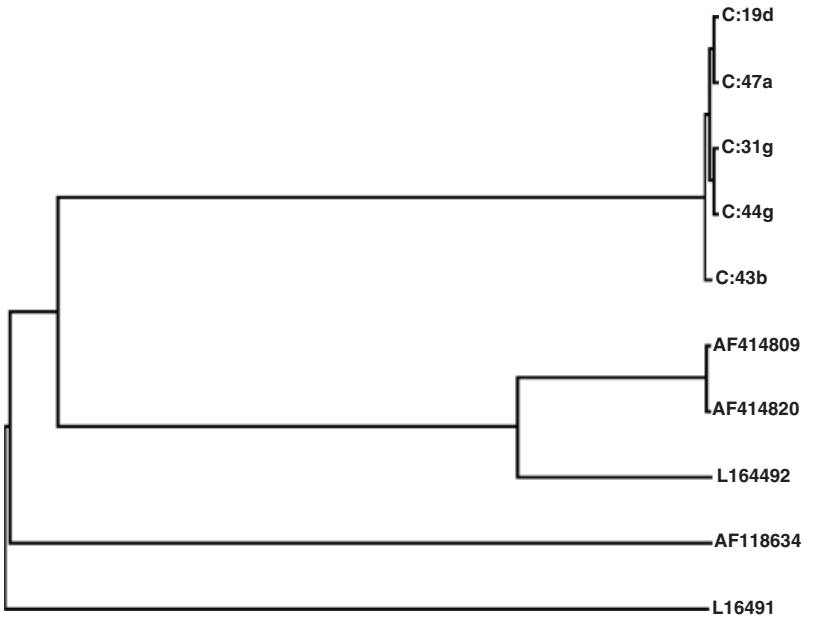

Fig. 1. Phylogenetic relationship between *fimA*-untypable clones and type I *fimA* clone. Patient samples (C:19d, C:47a, C:31g, C:44g and C:43b) of G3, which were not amplified with the *fimA* type-specific primers, but amplified with the 16S rRNA universal primer, were analysed. The 16S rRNA sequence of *Porphyromonas gingivalis* clones related to type I *fimA* (Accession Numbers: AF414809, AF414820 and L164492) was taken from the GenBank. The relationship among these clones was analysed as observed based on the matrix of pairwise distances.

Distribution of *fimA* genotypes

In all groups, the most prevalent *fimA* types were I, Ib and II, but with different distributions in each group. Type I *fimA* was the most frequently detected genotype individually (40%) and in its different combinations (40%) in the control group (G1; Table 2). In non-T2DM subjects with periodontitis (G2), the most frequently detected clone was type Ib *fimA* individually (20%) and in its different combinations (36%). In T2DM patients with periodontitis (G3), the most frequently detected individual types were I (20%) and III (20%). When types I, Ib and III were combined with other genotypes, their detection frequencies were 36%, 32% and 4%, respectively.

Table 2. Distribution of *Porphyromonas gingivalis fimA* genotypes detected individually or in combination with other types

fimA type	Group 1 % (f)	Group 2 % (f)	Group 3 % (f)
I	40 (10)	12 (3)	20 (5)
Ib	4 (1)	20 (5)	8 (2)
II	8 (2)	16 (4)	12 (3)
III	4 (1)	4 (1)	20 (5)
IV	0 (0)	4 (1)	0 (0)
V	0 (0)	0 (0)	4 (1)
I, Ib	8 (2)	28 (7)	24 (6)
I, Ib, II	12 (3)	4 (1)	4 (1)
I, Ib, II, IV	8 (2)	0 (0)	0 (0)
I, Ib, III, IV	4 (1)	0 (0)	0 (0)
I, Ib, V	4 (1)	0 (0)	4 (1)
I, II	0 (0)	4 (1)	0 (0)
I, III	0 (0)	0 (0)	4 (1)
I, IV	0 (0)	4 (1)	0 (0)
Ib, II	0 (0)	4 (1)	0 (0)
II, IV	4 (1)	0 (0)	0 (0)
I, II, IV	4 (1)	0 (0)	0 (0)
	100 (25)	100 (25)	100 (25)

Differences in distribution of *fimA* genotypes

Table 3 depicts the comparison of distributions of *fimA* genotypes between groups. There was a significant statistical difference ($p<0.05$) between periodontitis (G2 and G3) and non-periodontitis (G1) groups in distribution of type I, which was associated with healthy periodontal condition. In the groups affected by periodontal disease (G2 and G3) the distribution of types I and III *fimA* were more prevalent in T2DM patients with periodontitis, but there was no significant statistical difference ($p>0.05$).

Table 3. Differences in the total distribution of *Porphyromonas gingivalis fimA* genotypes.

fimA type	G1 *versus* G2	G1 *versus* G3	G2 *versus* G3
I	0.0366	0.0366	0.9999
Ib	0.2575	0.7745	0.3961
II	0.5443	0.2077	0.5078
III	0.5515	0.2214	0.0504
IV	0.2214	0.0184	0.1489
V	0.3124	0.5515	0.1488

1 G1, non-T2DM subjects without periodontitis; G2, non-T2DM subjects with periodontitis; G3, T2DM patients with periodontitis; *p*, χ^2 test or Fisher's Exact Test.

Discussion

The relationship between periodontal disease and DM provides an example of systemic disease pre-disposing to oral infection, and once that infection is established, the oral infection may exacerbate systemic disease by affecting its glycaemic control (Pickup & Crook 1998).

The detection of *P. gingivalis* was carried out by amplification of the 16S rRNA gene because this method has a higher sensitivity and specificity than traditional techniques such as bacterial culture (Riggio et al. 1996). In this study, the prevalence of *P. gingivalis* was 38% in subjects without periodontitis, which was similar to reports from Japan (36.3%; Amano et al. 2000) but higher than Brazil (17.7%; Amano et al. 2000, Missailidis et al. 2004). There are also differences in *P. gingivalis* detection in subjects affected by periodontitis, the prevalence being 70% in this study, while in Japan it was 87.1% and 89.4% in Brazil. These findings could be explained as differences in oral hygiene conditions, sampling site, periodontal tissue conditions, age and/or ethnicity of the populations studied (Haffajee et al. 2004). Besides, it was suggested that in some cases, *P. gingivalis* colonization is a contributory but non-essential factor for the progression of periodontal disease (Cullinan et al. 2001). On the other hand, it has been reported that in periodontitis, there is a mixture of bacteria organized for metabolic efficiency, greater resistance to stress and for enhanced virulence than those present in *P. gingivalis* alone (Marsh 2005).

In this study, the lateral incisor was selected because this tooth showed the highest value of LAL and PPD. This tooth-specific sampling method was used as a representative of the patient's mouth to avoid variations. The distribution of *fimA* genotypes of *P. gingivalis* in non-T2DM patients without periodontitis was quite similar to other reports in different populations around the world, where type I individually or in a combination, was associated with healthy periodontal tissues (Amano et al. 2000). In this study *fimA* type I (40%) was individually associated with healthy periodontal tissues, with similar data reported from Japan (50.7%; Nakagawa et al. 2002), but quite different results found in a Brazilian population where type I was found to be associated only with type IV (Missailidis et al. 2004). These differences from the Brazilian study can be explained as occurring because subgingival sampling was from different sites of the mouth and there was a mixture of patients affected with chronic and aggressive periodontitis. This last study also reported a high frequency of untypable samples (17.8%). In the present study, untypable samples that were negative for *fimA* genotypes but positive for *P. gingivalis* were further analysed to determine their genotypes by sequencing of 16S rRNA. The genotypes of all clones in those samples seemed to be very closely related to type I *fimA*, suggesting an increase in frequency of type I alone or in combination with other types. This means that type I *fimA* was often underestimated in other studies.

Most clinical studies agree that type II *fimA* is the most predominant type in patients affected by periodontitis, followed by genotype Ib (Amano et al.

2000, Nakagawa et al. 2002, Miura et al. 2005, Ojima et al. 2005); most of these studies were carried out in Japan. However, in a multiracial population such as in the Brazilian study, the most predominant type was Ib (Missailidis et al. 2004), this finding agreeing with our results. These data strongly suggested that ethnic and demographic variables are important to consider when final conclusions among cross-population studies are being drawn.

Comparisons between groups about combined distribution of *fimA* genotypes (Table 3) showed that there was an association for healthy periodontal tissues with genotype I, which had a significant statistical difference from T2DM subjects affected by periodontal disease. When the total distribution of single and combined *fimA* types of *P. gingivalis* was analysed in this study, the results show that for non-T2DM subjects without periodontitis the most prevalent types were I and II, while for non-T2DM subjects with periodontitis they were Ib, II and I, and for T2DM patients with periodontitis they were types I, III and II (Table 2). As Ib and II *fimA* genotypes of *P. gingivalis* are considered to be virulent variants, while strains that belong to types I and III were reported as being less virulent (Amano et al. 2000, 2004, Missailidis et al. 2004). These findings suggest than other factors such as altered innate immune response, microvascular impairments and the presence of advanced glycation end products should be considered to explain the high prevalence of periodontitis in T2DM patients (Joshi et al. 1999, Patiño-Marin et al. 2005, Takeda et al. 2006).

We conclude that type I was more frequently detected in periodontally healthy sites from non-T2DM subjects than in periodontitis sites from either subjects with or without T2DM. Type Ib was more frequently detected in periodontitis sites from non-T2DM subjects. However, in sites affected by periodontitis from T2DM subjects, the predominating types were I and III, which are less virulent strains of *P. gingivalis*.

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Clinical Relevance

Scientific rationale for study: Periodontitis is considered to be the sixth complication of DM, *P. gingivalis* considered as the main aetiological agent. There are no reports regarding the distribution of *P. gingivalis fimA* genotypes in a population with a high prevalence of T2DM.

Principal findings: In T2DM patients with periodontitis types I and III were more prevalent, but there was no statistical difference ($p > 0.05$) from non-T2DM periodontitis subjects. *P. gingivalis* clones with low virulence could lead to periodontitis in a population with high prevalence of DM.

Practical implications: Antimicrobial strategies for T2DM patients may need to differ from non-diabetic patients, given the widely recognized host defence compromises and the differences in *P. gingivalis* colonization patterns.