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Genotypic characterization of *Porphyromonas gingivalis* isolated from subgingival plaque and blood sample in positive bacteremia subjects with periodontitis

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Abstract

Aim: The objective of this study was to investigate clonal relationship among *Porphyromonas gingivalis* isolated from subgingival plaque and blood samples in positive transient bacteremia subjects with periodontitis.

Material and Methods: Unrelated patients with general chronic periodontitis or general aggressive periodontitis requiring scaling and root planing (SRP) were included in the study. Genotyping of each isolate was performed using pulsed field gel electrophoresis technique. Genetic relatedness of strains isolated within an individual or between different patients was determined by dendogram analysis.

Results: Following SRP, from 16 patients, seven patients showed positive *P. gingivalis* bacteremia and nine were negative. Thirty-two strains were isolated from subgingival plaque and blood samples before and during induced transient bacteremia. The majority of the patients harboured one clonal type. Two patients showed different clones in plaque and blood samples suggesting that more than one clone can be found in subgingival plaque. *P. gingivalis* isolates from periodontitis patients after transient bacteremia following SRP, revealed a high heterogeneity among isolates.

Conclusion: In 6/16 subjects the same *P. gingivalis* isolate was found in the blood and in oral cavity. *P. gingivalis* heterogeneity suggests no association of a unique clonal type with transient bacteremia.

Key words: bacteremia; genetics; periodontitis; *Porphyromonas gingivalis*; pulsed field gel electrophoresis

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Porphyromonas gingivalis belongs to the *Porphyromonacea* family and *Bacteroidetes* phylum. *P. gingivalis* is a Gram-negative, anaerobic, but aerotolerant, assacharolytic, β-haemolytic, non-motile, non-sporiform, black pigmented rod. *P. gingivalis* is considered an opportunistic pathogen that is infrequently isolated in healthy sites, but increases in prevalence and proportion in actively destructive periodontal disease sites. Thus, it has been described as a major etiological agent in the onset and progression of chronic destructive periodontitis (Haffajee et al. 1997, Ting & Slots 1997).

P. gingivalis has been isolated from both oral and occasionally in extra-oral sites. For example it has been isolated from tubal-ovarian abscesses (Hirata et al. 1995) and pulmonary abscesses

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(Shah et al. 1976), but also during orally induced bacteremia (Forner et al. 2006, Lafaurie et al. 2007). A strong link has been established between periodontal disease and systemic complications (Lourbakos et al. 2001) including cardiovascular disease, stroke, lung inflammation, and preterm low weight birth (Scannapieco et al. 1992, Cueto et al. 2005, Offenbacher et al. 2006, Renvert et al. 2006). In addition *P. gingivalis* identification has been made by molecular technique in atheromatous plaques (Kozarov et al. 2006, Padilla et al. 2006, Pucar et al. 2007). Although bacteremia by *P. gingivalis* is a new field of study, a recent report has shown that experimental *P. gingivalis* bacteremia can induce aortic and coronary lesions consistent with atherosclerosis in hypercholesterolemic pigs (Brodala et al. 2005).

In this study, *P gingivalis* was detected by polymerase chain reaction (PCR).

Today, traditional methods of strain typing, including bacteriophage typing and serotyping, have been replaced by newer molecular methods. Molecular typing systems are able to distinguish among epidemiologically unrelated isolates by characterizing the genetic variations in the chromosomal DNA of bacterial species. Several DNA-based typing methods have been used for characterizing *P. gingivalis*: e.g., random amplified polymorphic DNA (Menard & Mouton 1995), restriction fragment length polymorphism analysis (Loos et al. 1993), DNA fingerprinting by amplified fragment length polymorphisms (Ozmeric et al. 2000), restriction enzymatic analysis (REA) (Rijnsburger et al. 2007), and pulsed field gel electrophoresis (PFGE) (Hirata et al. 1995, Nakayama 1995). Nevertheless, PFGE and multilocus sequence typing (MLST) are the two methods most commonly used to discriminate among isolates below the species level.

Studies performing genotyping have provided evidence for significant *P. gingivalis* intraspecific heterogeneity (Loos et al. 1990, Menard et al. 1992, Chen & Slots 1994, Ozmeric et al. 2000). However, the clonal relationship between strain from subgingival plaque and blood samples during bacteremia has not been studied. Bacteremia occurs when bacteria reach blood stream transiently and can be detected by laboratory blood cultures technique (Kinane et al. 2005). *P. gingivalis* transient bacteremia following SRP procedure is the most frequently bacteremia (Lafaurie et al. 2007). However, the genetic relatedness between isolates from subgingival plaque and blood stream samples has not been studied. The aim of this study was to determine by PFGE assessment the genetic relatedness between *P. gingivalis* isolated from subgingival plaque and blood samples before and during transient induced bacteremia following SRP procedure in order to determine if a genetic trend is associated with the ability of *P. gingivalis* to survive in blood during bacteremia after SRP.

Material and Methods

Population

Sixteen unrelated systemically healthy adults were included in this study. They were selected from a total of thirty-two subjects who attended the Graduate Clinic of Periodontics and Oral Medicine Service of El Bosque University in Bogotá (Colombia). All subjects were diagnosed with either adult generalized chronic periodontitis (10/16) or generalized aggressive periodontitis (6/16). Patients must have had at least 10 pockets with probing depth $\geqslant 7$ mm requiring periodontal surgery after scaling and root planing (SRP). All patients were positive for *P. gingivalis* in subgingival plaque sample before the SRP. In addition laboratory exams, e.g., glucose test and haemogram, were made in order to discard patients with medical compromise. The Ethics Committee of El Bosque University approved this study, and all patients provided informed consent. All patients were diagnosed according to criteria established by the American Association of Periodontics in 1999 (Armitage 1999). Clinical history was evaluated and radiographic examinations were performed for each patient. Periodontal examination included the assessment of pocket depth, loss of attachment and bleeding. The exclusion criteria for this study were applied as described previously (Lafaurie et al. 2007).

Samples collection

Samples were collected from subgingival plaque and from blood for analysis (Lafaurie et al. 2007). Briefly, a subgingival study inclusion sample was collected from six pockets (>7 mm). The surgical day, a second subgingival sample was collected before SRP procedure. Six sites were targeted for the first sample and four more sites were selected according to pocket depth and easy access for treatment for the second sample. Subgingival plaque samples were taken using sterile paper points and then pooled. The delay between the two samples was 1–4 weeks. Four blood samples were collected from the antecubital vein at the following time points: (H1) before the SRP procedure; (H2) immediately at the end of the procedure; (H3) 15 min. after and (H4) 30 min. after the end of SRP procedure. SRP was assessed during 10 min. (10 sites, 1 min. per site). All patients were instructed to not have breakfast or teeth brushing before the procedure to avoid another source of bacteremia different than the SRP.

P. gingivalis identification

The following tests were performed to positively identify and confirm the *P. gingivalis* strain: Colony morphology, (carbobenzoxy-L-arginin-7-amino-4-metylcoumarin, Sigma, St. Louis, MO, USA) positivity (Slots 1987), the commercially available rapid-ID 32A system (*Biomerieux*, Lyon, France), and 16 s RNA-based PCR using primers described by (Ashimoto et al. 1996).

P. gingivalis growth conditions

P. gingivalis were cultured on 4.5% Brucella agar (BBL Microbiology Systems, Cockeysville, MD, USA.) enriched with 0.3% Bacto Agar (Difco, Detroit, MI, USA), 0.2% yeast extract, 5% defibrinated sheep blood, 0.2% haemolyzed sheep red blood cells, 0.0005% haemin (Sigma), and 0.0001% menadione (Sigma). Samples were incubated at 36°C in an anaerobic atmosphere (Anaerogen, Oxoid, Hampshire, UK) for 5 days.

Preparation of DNA for PFGE

DNA samples were prepared in agarose gel by a novel protocol standardized by UIBO laboratory (Basic Oral Research Unit Institute). Briefly, *P. gingivalis* was recovered from solid culture and suspended in a solution of 10 mM Tris HCl (pH 7.6) and 1 M NaCl (Nakayama 1995). After washing twice in the same buffer, cells were suspended at $OD_{650\,nm} = 1.3–1.4$. The bacterial suspension was mixed with 1% of low melting temperature agarose (Bio-rad, Hercules, CA, USA). The agarose was solidified in a reusable plug mould (Bio-

rad) for 30 min. at 4°C. The plugs (0.8 cm × 0.8 cm) were treated at 37°C for 15 h in 1 ml of solution containing 1 M Tris HCl (pH 7.6), 1 M NaCl, 0.5 M EDTA (pH 8), sodium deoxycholate (0.2%), lauroyl sarcosinate (0.5%), lysozyme (4.37 mg/ml), and RNase (20 μg/ml). After that, the plugs were washed once with the same buffer and incubated at 50°C for 48 h in 2 ml of solution containing EDTA (0.8%), SDS (1%), and protease (2 mg/ml). Then plugs were washed three times for 2 h with a solution containing 10 mM Tris HCl (pH 7.5) and 0.1 mM EDTA at room temperature. The enzyme restriction assay was performed by adding *Not*I (Nakayama 1995) to plugs in 200 μl of restriction reaction buffer for 4 h following Promega (R6435) instructions. Each experiment was repeated three times.

PFGE

After the enzyme restriction treatment, plugs were inserted into the lanes of a 1% pulse field certified agarose (Bio-rad) gel. PFGE was performed in a contour-clamped homogeneous electric field DR III apparatus (CHEF DR III; Bio-rad). Electrophoresis buffer was a tris borate buffer (TBE, 0.5 ×, pH 8). Electrophoresis conditions were: voltage 60 V, field angle 120°, temperature 11°C, running time 24 h, initial pulse 5.5 s, and final pulse 49.9 s. Lambda marker (Biolabs, New England, Biolabs, Beverly, MA, USA) was used to indicate standard molecular weights. Following electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml; Sigma), and visualized with a transluminator. Reproducibility of the typing technique was validated during the standardization of the PFGE technique. This step was made with the reference strain *P. gingivalis* ATCC33277.

Bioinformatics analysis

PFGE profiles were analysed by using the GelCompar software package (Applied Maths, Kortrijk, Belgium). Cluster analysis was performed with the unweighted pair group method with arithmetic mean (UPGMA) analysis based on a Dice coefficient. Two isolates were considered genetically related if their Dice coefficient was equal to 85% or more. The cut off level of 85% was validated when two isolates representing the same bacterial population found at two different times showed a different PFGE pattern consistent with a single genetic event.

Results

Population and *P. gingivalis* strains classification

A group of 16 Colombian adults with periodontitis were studied. Patient demographic data and clinical data are showed in Table 1. Patients were divided in two groups based on the location of positively identified *P. gingivalis* strains: Group 1 (seven patients-17 strains) scored positive in samples from the oral cavity (subgingival plaque) and the blood (*P. gingivalis* positive bacteremia) (Table 2); Group 2 (nine patients-15 strains) scored positive only in samples from the oral cavity (*P. gingivalis* negative bacteremia).

PFGE

PFGE was used to identify the phylogenetic relationships among 32 *P. gingivalis* strains isolated from subgingival plaque and blood samples after orally induced bacteremia. The relationships between the PFGE patterns (Fig. 1) were expressed as percentages of similarity. Results for Group 1 (positive bacteremia) are listed in a dendogram in Fig. 2.

In Group 1, the dendogram revealed that from 17 strains (seven patients); five strains found in the blood were the same clones as those found in the oral cavity (patients 1, 2, 4 and 5). Two strains found in the blood were not the same clones as those found in the oral cavity (patients 3 and 6) (Fig. 1, lanes 2 and 3 for patient 3 and lanes 8 and 9 for patient 6). Due to the non-ability of process subgingival plaque samples for patient 7, we could not compare strains from the blood and subgingival samples. However, the strains found in different blood samples of patient 7 (UIBO 486 H2 and UIBO 486 H3) were from the same clone. Similarly, the strains found in the blood of patient 1 (UIBO 655 H3, UIBO 655 H4) were from the same

Table 1. Age, gender and clinical parameters in patients treated with scaling and root planing

Parameter	Generalized chronic periodontitis		Generalized aggressive periodontitis	
	total	per cent	total	per cent
Subjects	10/16	62.5	6/16	37.5
Gender (%)	F	M	F	M
	6	4	4	2
Age (mean ± SD)	45.2 ± 7.9		32.6 ± 4.2	
PD (mean ± SD)	3.5 ± 0.7		4.1 ± 0.6	
CAL (mean ± SD)	3.4 ± 1.3		5.2 ± 1.3	
Bleeding on probing (% positive)	65.2 ± 27.6		74.2 ± 21.3	
PD treated sites with SRP mm (mean ± SD)	7.5 ± 1.8		8.1 ± 1.6	

CAL, clinical attachment level; PD, probing depth.

Table 2. Patient population and *Porphyromonas gingivalis* strains from UIBO (Basic Oral Research Unit Institute) isolated in transient bacteremia

Patient	*P. gingivalis* isolates				
	in subgingival plaque sample		in blood sample*		
	inclusion study sample	before scaling and root planing	H2	H3	H4
1	NA†	UIBO 655		UIBO 655 H3	UIBO 655 H4
2	NA	UIBO 695	UIBO 695 H2		
3	NA	UIBO 728		UIBO 728 H3	
4	UIBO 735	UIBO 771	UIBO 771 H2		
5	UIBO 751	UIBO 801		UIBO 801 H3	
6	NA	UIBO 1047		UIBO 1047 H3	
7	NA	NA	UIBO 486 H2	UIBO 486 H3	

*Blood samples collected during transient bacteremia following SRP procedure: immediately at the end of the procedure (H2); 15 min. after (H3) and 30 min. after (H4) the end of the procedure.
†NA, not able to genotype (but culture positive).
SRP, scaling and root planing.

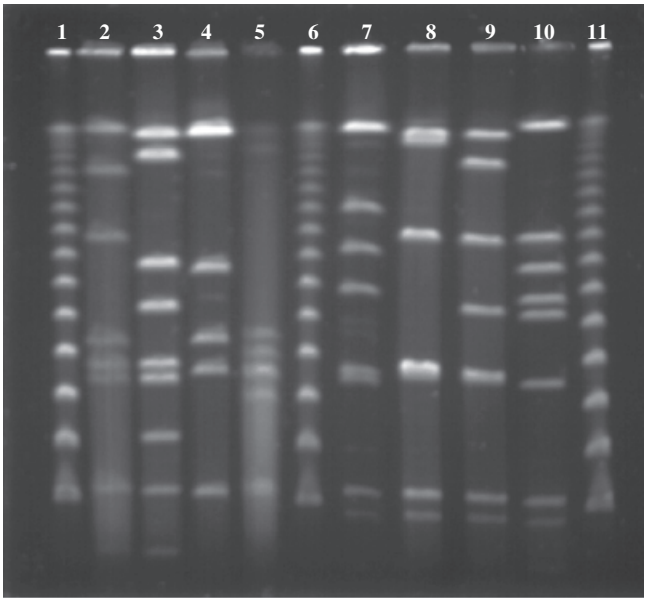

Fig. 1. PFGE analyses of *Not*I digested genomic DNA of *P. gingivalis* strains. Lanes 1, 6 and 11, Lambda DNA ladder; lane 2, UIBO *728; lane 3, UIBO 728 H3; lane 4, UIBO 751; lane 5, UIBO 771; lane 7, 472; lane 8, UIBO 1047; lane 9, UIBO 1047 H3; lane 10, ATCC 33277 (American Type Culture Collection 33277, reference strain). **P. gingivalis* strains preceded by UIBO indicate that those strains belong to the Basic Oral Research Unit Institute (UIBO) strain collection. PFGE, pulsed field gel electrophoresis.

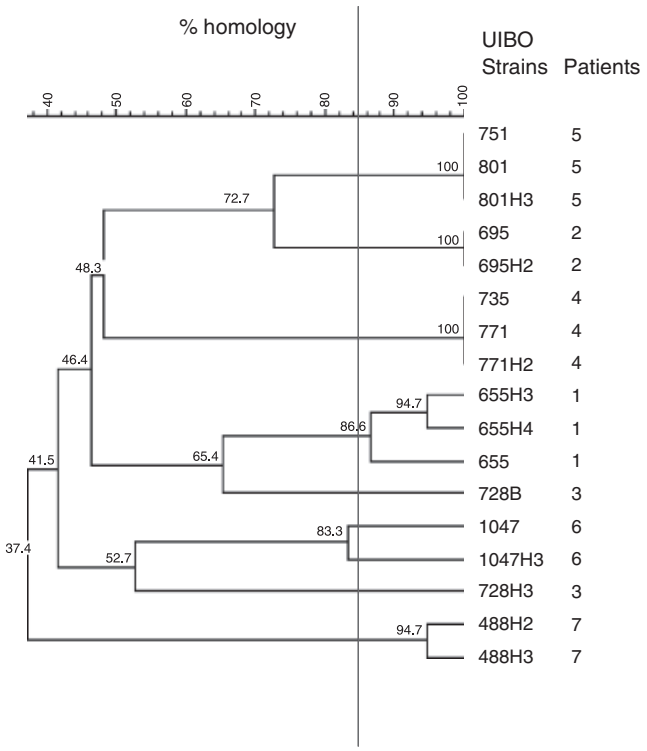

Fig. 2. Dendogram representing the genetic relationships between strains of *P. gingivalis*. Cluster analysis was performed with the UPGMA analysis based on a Dice coefficient. For a Dice coefficient $\geqslant 85\%$, the isolates were considered genetically related. UPGMA, unweighted pair group method with arithmetic mean.

clone. Thus, we did not find different clones in strains from the blood of a single patient; but different clones were found in strains from the oral cavity and blood of a single patient.

In Group 2 (data not show), concerning oral strains collected in inclusion study sample and at the same day of the procedure in the same patient (12 strains), eight strains are the same clone within individual patients (patients 11, 12, 15 and 16). Conversely, four strains from patients 13 and 14 are not the same clone and do not belong to the same cluster.

Discussion

We analyzed a total of 32 *P. gingivalis* isolates from a group of unrelated Colombian adults with periodontitis. Results indicate there is a great diversity among *P. gingivalis* strains; the PFGE profile showed a different clonal type in each subject. Thus there is a non-clonal population supporting the high diversity among *P. gingivalis* strains, such as previously identified by others using different genotyping methods including DNA fingerprinting, MLEE, REA and AP-PCR (Loos et al. 1990, 1993, Menard et al. 1992, Chen & Slots 1994, Menard & Mouton 1995). The diversity in the *P. gingivalis* panmictic population is due to recombination as described by (Frandsen et al. 2001), (Koehler et al. 2003) and (Enersen et al. 2006). For the majority of the patients, (12/16), each individual carried only one clonal type in subgingival plaque. However, for a minority of the patients, (4/16), each individual harboured two clones. These results are in agreement with those previously reported by (Loos et al. 1990, 1992), van Steenbergen et al. (1993), and (Leys et al. 1999); they found a prevalence of only one clonal type in individual subjects in the study population when assessing clonal diversity among *P. gingivalis* strains by different genotyping methods (DNA fingerprinting, REP and heteroduplex analysis). The results were similar in a large sample population of 661 patients (Leys et al. 1999) and in a small sample population of nine patients (Loos et al. 1992). The finding of several clones in the same patient was more recently described by (Enersen et al. 2006); they found two, three, or four STs (sequence type) in three Indonesian patients by MLST typing. Previously, (Loos et al. 1990) and (van Steenbergen et al. 1993) reported that not more than

two clonal types of *P. gingivalis* were found in individuals from Western populations.

We did not find a unique clonal type of *P. gingivalis* associated with generalized periodontitis. Therefore different clonal types were associated with generalized periodontitis. This result is in accordance with reports by (Loos et al. 1992) and Menard & Mouton (1995), who found different clones in subgingival plaque samples. Regarding the strains found in the blood and oral cavity within one patient, five strains found in blood were from the same clone of oral cavity (patients 1, 2, 4 and 5). Considering that SRP is a common professional treatment, this study provides molecular evidence that bacteria located in the oral cavity may reach the bloodstream after the SRP procedure. Therefore, this study suggests there is a relationship between *P. gingivalis* from oral cavity, bacteremia and extra oral infections. Thus the prevention of periodontal disease is important in preventing both orally induced bacteremia and its systemic complications related to periodontal disease.

Nevertheless, two patients harboured different clones in oral cavity and blood; this finding suggests that those patients could harbour two different clones in subgingival plaque and that we only identified one. Although this finding suggests that clonal type identified from blood does not always reflect the clonal pattern from subgingival plaque samples.

Individual clonal types in the oral cavity appear to be constant for a period of 1–4 weeks, as indicated by the comparison of *P. gingivalis* strains isolated in subgingival plaque samples the same day of the procedure and those identified on the day of enrollment in the study. In some cases we found different clones in the two samples; this can be explained by the emergence of another clone of *P. gingivalis* during the period of sampling. Therefore the clone found at the time of inclusion in the study was replaced by the one identified in the subgingival sample on the day of the procedure. The latter clone was most likely present in a family member (e.g., spouse) and transmitted by direct contact for further, stable colonization (Saarela et al. 1993, van Steenbergen et al. 1993).

In conclusion, this study was the first to investigate clonal variability in *P. gingivalis* isolated from a group of Colombian patients with periodontitis before and during orally induced transient bacteremia. This study showed unique patterns of *P. gingivalis* for each subject and therefore the absence of a single clone that could be identified specifically with generalized periodontitis or *P. gingivalis* transient bacteremia**.** This suggests there is an extensive heterogeneity within the *P. gingivalis* species. Moreover, we confirmed the presence of no more than two clones of *P. gingivalis* in individual patients in a Southwestern population, i.e., we found low heterogeneity among *P. gingivalis* isolated within a single individual. Finally, for the first time our data demonstrate that there is a tendency for clonal homology in *P. gingivalis* strains in bloodstream samples and subgingival plaque samples in an individual subject. Nevertheless, our results indicate that further studies involving *P. gingivalis* and orally induced transient bacteremia must consider the potential of clonal diversity within one patient.

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Clinical Relevance

Scientific rationale for the study: *P. gingivalis* is the periopathogen more frequently isolated from blood during bacteremia after SRP. Therefore, in order to determine if a genetic trend is associated with the ability of *P. gingivalis* to survive in blood during bacteremia after SRP, it is necessary to study the genetic relatedness between strains isolated from subgingival plaque before SRP and blood samples isolated during transient bacteremia.

Principal findings: *P. gingivalis* isolated from subgingival plaque and blood sample among periodontitis patients shows high heterogeneity. The majority of the patients shows *P. gingivalis* homology from subgingival and blood samples. However, it is possible to find different clones between both samples in the same patient.

Practical implications: Unrelated patients with periodontitis are infected by different *P. gingivalis* clones. Strains found in blood samples during transient bacteremia following SRP usually have a genetic homology with the ones isolated from subgingival plaque sample. Finally, the genetic relationship between strains found in subgingival plaque and blood samples within individual patient increases the evidence about the impact of periodontal infection in extra-oral infections and systemic diseases.