Detection of Periodontal Bacteria in Atheromatous Plaques by Nested Polymerase Chain Reaction

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Background. In recent years, increasing evidence regarding the potential association between periodontal diseases and cardiovascular diseases has been elicited. The available evidence underlines the importance of detecting periodontal pathogens on atheromatous plaques as the first step in demonstrating the causal relationship between both entities. It is the main aim of this investigation to detect periodontitis-associated bacteria from carotid artery atheromatous plaques recovered from patients who received an endarterectomy, using strict sample procurement and laboratory procedures. **Methods.** Atheromatous plaques from endarterectomies from carotid arteries were scraped, homogenized and bacterial DNA was extracted. In order to obtain a representative concentration of amplicons, two amplifications of the bacterial 16S ribosomal-RNA gen were carried out for each sample with universal eubacteria primers by polymerase chain reaction (PCR). Nested-PCR with specific primers for the target bacteria was performed next. Statistical tests included χ^2 test. **Results.** Forty-two atheromatous plaques were analyzed. All of them were positive for at least one target bacterial species. The bacterial species most commonly found in atheromatous plaques was *Porphyromonas gingivalis* (78.57%, 33/42), followed by *Aggregatibacter actinomycetemcomitans* (66.67%, 28/42), *Tannerella forsythia* (61.90%, 26/42), *Eikenella corrodens* (54.76%, 23/42), *Fusobacterium nucleatum* (50.00%, 21/42) and *Campylobacter rectus* (9.52%, 4/42). The simultaneous presence of various bacterial species within the same specimen was a common observation. **Conclusion.** Within the limitations of this study, the presence of DNA from periodontitis-associated bacteria in carotid artery atheromatous plaques retrieved by endarterectomy was confirmed.

KEY WORDS

periodontal diseases, cardiovascular diseases, atherosclerosis, *Porphyromonas gingivalis*, polymerase chain reaction, microbiology

Atherosclerosis is the major event in the pathophysiology of cardiovascular diseases (CVD), where large to medium-size muscular and large elastic arteries become occluded with fibro-lipidic lesions, known as atheromas. These atheromatous plaques are responsible for end-stage complications or events associated to CVD, such as coronary thrombosis, acute myocardial infarction and stroke[1].

In recent years, increasing evidence regarding the potential association between periodontal diseases and CVD has been elicited. In fact, recent meta-analysis have shown that this association is statistically significant and consistent, although of a low magnitude[2-4]. These studies support the hypothesis that periodontitis may confer an independent risk to CVD. Due to the high prevalence of periodontitis in humans, and due to the fact that CVD are the main cause of death in developed countries, an increasing interest has raised in the scientific community to elicit which are the potential links between both entities[5, 6].

Several hypotheses have been proposed to explain why periodontitis may increase the risk of CVD[6, 7]; from an indirect association determined by common risk factors or a common phenotype underlining both conditions[8]; to a direct association between the periodontal infection and the pathophysiology of the atherosclerotic lesion[9]. This last hypothesis is supported by evidence that bacterial pathogens derived from the subgingival biofilm might be directly or indirectly (through the resulting host response) involved in the process of atherogenesis[5]. A possible common predisposition underlying both diseases, however, might co-exist with the direct influence of periodontitis and CVD[6]. Periodontitis is a chronic "inflammatory" disease of multifactorial aetiology, being the primary aetiological factor the presence of specific bacteria residing in the subgingival biofilm. This subgingival biofilm is a complex microbiota where more than 700 bacterial species have been detected[10]; however, only a limited number of these bacteria has been shown to be a risk factor for the initiation or progression of periodontitis, namely *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia*[11] . The presence of these periodontal pathogens has been demonstrated not only associated with disease sites, but also in healthy sites albeit in low numbers. Its frequency of detection also varies in different geographical locations, being for example the prevalence of *P. gingivalis* higher in Spain than in other countries, as in The Netherlands[12].

These periodontal pathogens present in the subgingival biofilm have also been identified in the blood of patients with periodontitis, mostly associated with periodontal interventions, although also after normal day-life activities such as mastication[13], tooth brushing[14] or dental flossing[15]. The occurrence of these bacteremias raises the hypothesis of bacterial colonization at distant sites and their specific involvement in the pathogenesis of the atherosclerotic lesion. In fact, experimental studies have shown the potential ability of these pathogens of being involved in different stages of the development of the atherosclerosis lesion, mainly by: (a) favoring the adherence of leukocytes to the vascular endothelium by increasing the expression of vascular cellular adhesion molecule (VCAM)-I, intracellular adhesion molecule (ICAM)-I and E-selectine in human aortic endothelial cells[16]; (b) favoring the migration of monocytes through the expression of monocyte chemo-attractant protein (MCP)-I in endothelial cells infected with *P. gingivalis*[17]; (c) promoting the transformation of macrophages into foam cells[18, 19]; (d) having a pro-coagulant effect[20]; (e) favoring the rupture of the atheromatous plaque through the release of metalloproteinases[21]. This experimental evidence, although in vitro, underlines the importance of detecting and identifying these putative pathogens on atheromatous plaques as the first step in understanding the possible associations between periodontitis and CVD.

The identification of periodontal bacteria DNA in atheromatous plaques was first reported in 1999 from samples of human carotid endarterectomies[22]. Since then, other similar investigations have reported conflicting results, which may be attributed to differences in the procurement of the specimens or to differences in the molecular technique used for the bacterial DNA identification[23-29]. The expected amount of bacteria is small and since atheromatous plaques are complex lesions to extract DNA there is a need for strict laboratory protocols aimed for a sensitive and specific detection of bacterial DNA[30, 31]. It is, therefore, the aim of this investigation to detect DNA from periodontitis-associated bacteria in carotid artery atheromatous plaques recovered from patients who received an endarterectomy using strict sample procurement and laboratory procedures. It is our hypothesis that bacterial DNA from periodontopathic bacteria would be present in the retrieved atherosclerosis samples and this presence will be related to the oral health status of the patients.

MATERIAL AND METHODS

Sample

The study sample consisted on atheromatous plaques retrieved during endarterectomy surgical procedures from the carotid artery of consecutive patients admitted to the Department of Angiology and Vascular Surgery from University Central Hospital Asturias (HUCA) in Oviedo (Spain), due to various manifestations of cardiovascular disease (symptomatic patients with stenosis greater than 70% or asymptomatic patients with pre-occlusive stenosis). The inclusion period was from December 2006 to January 2008. Patients fulfilling the inclusion criteria were informed of the scope of the study and signed an informed consent form previously approved by the Ethics Committee of HUCA (Oviedo, Spain). The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Specimen Collection

All specimens were dissected in the operating room and placed in transport vials under sterile conditions. Samples were immediately frozen at -20ºC and they were sent to the Research Laboratory of the Faculty of Dentistry, University Complutense, Madrid, Spain, by a special courier service that maintained the temperature of -20ºC until processing.

Specimen Homogenization

Transport vials were opened at the research laboratory with care of maintaining aseptic handling of the specimens. The inner part of the plaques was scraped with a sterile blade, weighted and transferred to 15 ml sterile plastic tubes with suspension buffer[¶]. Specimen homogenization was achieved with a mechanical homogenizer[#] until a uniform suspension was obtained.

DNA Extraction

Total bacterial DNA from the whole homogenized samples was extracted by using a commercial kit[¶]. In order to obtain a more purified DNA, an additional final step with phenol:chloroform:isoamyl alcohol solution (1:4:24) was added to the protocol given by the manufacturer. Two ml phenol:chloroform:isoamyl alcohol solution were added and centrifuged at 9000xg for 15 minutes. The resulting supernatant was utilized and

2ml Tris-EDTA buffer was carefully added to the mixture. DNA was then precipitated with ice-cold pure ethanol and resuspended in 50µl of water[**].

Polymerase Chain reaction (PCR) Amplifications

First, an amplification of the bacterial 16S ribosomal-RNA (rRNA) gen was carried out with broad-range eubacterial primers (Forward: 5´- GAG TTT GAT CCT GGC TCA G -3´; Reverse: 5´- AGA AAG GAG GTG ATC CAG CC-3´). This PCR amplification was performed in a master mix solution containing 0.4 units of Taq DNA polymerase, 1x polymerase buffer with 2mM $MgCl_2$, 0.2 mM of a mixture of each deoxynucleoside triphosphate, 1µM primers and 10µl template DNA, in a total volume of 50µl. Samples were preheated at 95ºC for two minutes followed by an amplification under the following conditions: denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1 minute and elongation at 72ºC for 1 minute. Thirty-five cycles were performed followed by an elongation step at 72ºC for 10 minutes.

Duplicate reactions were prepared for each sample. The obtained PCR products were purified and concentrated with a purification kit[††] in a unique final solution.

Secondly, a nested PCR with specific primers for periodontal pathogens (*A. actinomycetemcommitans, P. gingivalis, Eikenella corrodens, Campylobacter rectus, T. forsythia* and *Fusobacterium nucleatum*) was performed. Primers were designed on the basis of 16S rRNA gen reported by Ashimoto et al. [32] (Table 1)

PCR amplification was performed in a master mix solution containing 0.2 units of Taq DNA polymerase, 1x polymerase buffer with 2mM $MgCl_2$, 0.2 mM of a mixture of each deoxynucleoside triphosphate, 1µM primers and 2µl PCR products as template DNA, in a total volume of 25µl. PCR conditions were the same as previously described for the broad-range eubacterial primers.

Negative and positive controls were included in each batch of samples. The negative control was sterile distilled water instead of template DNA. Positive controls consisted of genomic DNA isolated from cultures of reference bacterial strains (Table 2).

The detection limit of the nested PCR was assessed by determining the results (positive/negative) of serial 10-fold dilutions of extracted genomic DNA from each targeted bacteria. Dilutions ranged from 10^9 UFC/ml to 10^1 UFC/ml. Irrespective of the pathogen, the methodology allowed for the amplification of 10^2 UFC/ml.

The specificity of the procedure was tested for each set of primers with purified genomic DNA from each bacterium except the targeted one in each case. No cross-reaction was observed.

Analyses of PCR Products

A 12-µl aliquot of amplified sample from each PCR reaction was electrophoresed through a 1% agarose gel[‡‡] in Tris-acetate ethylenediaminetetraacetic acid (EDTA) buffer. The gel was stained with ethidium bromide (10mg/ml) and visualised under an ultraviolet light transilluminator[§§]. A DNA ladder[‖‖] was used as molecular weight marker. The band position of the PCR products was in accordance with the length of the primers.

Representative samples (two positive samples per bacteria) were sent for sequencing to a reference laboratory at the Faculty of Biology, University Complutense, Madrid, in order to confirm the obtained results.

Periodontal Examination

Once recovered from the endarterectomies, all patients were invited for a consultation obtaining demographic information, smoking habits and dental and medical history. Patients were then subject to a complete oral examination by a single trained periodontist that collected data on tooth loss, probing pocket depth and mobility at six sites per tooth, excluding third molars, using a CPC-12 periodontal probe[¶¶]. All patients who underwent the periodontal examination had a panoramic radiograph taken, where the percentage of bone loss was calculated. Based on the clinical and radiographical data, subjects were diagnosed of periodontitis or healthy/gingivitis [33].

Data Analysis

A subject-level analysis was performed for each study parameter. Data were expressed by means and standard deviations (SD) for all variables.

The frequency of pathogen detection in atheromatous plaques was obtained for each patient.

In addition, patients with periodontal data were stratified according to their number of teeth (edentulous, between 0-10, 11-20 and more than 20 teeth) and to the proportion of probing pocket depths (PPD) (1-3 mm or shallow, 4-6 mm or intermediate, >6 mm or deep). Differences in term of prevalence of pathogens in these subgroups were determined by χ^2 test. Statistical significance was established at the 95% confidence level. A statistical software package[##] was used for all data analyses.

RESULTS

Data From Atheromatous Plaques

42 atheromatous plaques were analysed.

The mean weight of the inner parts of atheromatous plaques that were analysed was 201.68 mg (SD=231.00). All the analysed samples were positive for at least one target bacterial species. The bacterium most commonly found in atheromatous plaques was *P. gingivalis* (78.57%, 33/42), followed by *A. actinomycetemcomitans* (66.67%, 28/42), *T. forsythia* (61.90%, 26/42), *E. corrodens* (54.76%, 23/42), *F. nucleatum* (50.00%, 21/42) and *C. rectus* (9.52%, 4/42).

The simultaneous presence of various bacterial species within the same specimen was a common observation (Table 3), with *A. actinomycetemcomitans* and *P. gingivalis* simultaneously present in 61.90% of the samples.

Patient-Based Data

From the 42 patients who underwent vascular surgery, 22 accepted to participate in the oral health consultation, where demographic and oral health information was obtained.

The patient's mean age was 71.1 years (ranging from 57-81). Half of the patients were surgically treated from a pre-occlusive asymptomatic stenosis and the other half from a stenosis greater than 70% symptomatic. Twelve patients were smokers (10-60

cigarettes per day) and ten were former smokers, eight patients had hypertension and six patients had diabetes.

From the 22 patients who underwent periodontal examination, 4 were edentulous, and therefore, they were excluded from the intra-oral data analysis. Patients referred tooth mobility as the main cause for tooth extraction; however, none of the patients had received previous periodontal treatment. The mean number of teeth present in the 18 dentate patients was 13.05 (SD=9.13) and the mean probing pocket depth was 4.47 mm (SD=0.83). The proportions of sites with probing pocket depth lower than 4 mm, 4-6 mm or deeper than 6 mm were 17.87% (SD=20.25), 77.85% (SD=18.80) and 4.28% (SD=7.69), respectively. The proportions of teeth with mobility grade I was 49.25% (28.76), grade II 28.65% (SD=21.75) and grade III 10.85% (SD=16.54). The average of bone loss measured in panoramic radiographs was 56%. The patients' diagnosis was either edentulous or suffering moderate to advanced chronic periodontitis.

Presence of Periodontal Bacteria in Atheromatous Plaques Stratified by Patients Groups

Dentate patients had significantly higher levels of *A. actinomycetemcommitans* in atheromatous plaques than edentulous patients. They also had a higher prevalence of *P. gingivalis, T. forsythia and E. corrodens* than edentulous patients, although these differences were not statistically significant. By contrast, edentulous patients had higher levels of *C. rectus and F. nucleatum* (Table 4).

When patients were stratified by the number of teeth present a positive linear trend with the prevalence of *A. actinomycetemcommitans* and *P. gingivalis* was observed (Table 4).

When the prevalence of bacterial species in atheromatous plaques was correlated with the patients' PPD distribution, it was observed that patients with PPD greater than 6 mm, presented *E. corrodens* and *F. nucleatum* more frequently than patients with PPD lower or equal to 6 mm. A clear trend was also seen for *P. gingivalis* and *T. forsythia,* although differences were not statistically significant (Table 5).

When the data on bacterial prevalence was compared between the patients that had the oral examination versus those who did not, differences were not significant (data not shown).

DISCUSSION

The present study aimed to investigate the presence of DNA from periodontal bacteria in atheromatous plaques retrieved from patients who had received endarterectomies due to various manifestations of ischemic vascular disease. These samples were subjected to meticulous laboratory procedures in order to optimise the sensitivity and specificity of detection. All of the analysed samples resulted positive for the DNA of at least one of the target bacterial species. DNA from bacteria most commonly found were *P. gingivalis* (78.57%), followed by *A. actinomycetemcomitans* (66.67%) (Table 6). The concomitant detection of DNA from *P. gingivalis* and *A. actinomycetemcomitans* was observed in 61.90% of the samples. These results are similar to those found by Gaetti-Jardim et al. [27], with 64.1% of clinical samples from periodontitis patients in which DNA from two or more species were detected.

Other authors have identified *P. gingivalis* and *A. actinomycetemcomitans* as the most prevalent DNA from bacteria in atheromatous plaques[24, 27, 29, 34]. By contrast, some reports were not able to detect *P. gingivalis*[23, 35], *A. actinomycetemcomitans*[36-39] or none of them[25, 26, 28, 40-42] (Table 6).

DNA from *T. forsythia* (61.90%), *E. corrodens* (54.76%), *F. nucleatum* (52.38%) and *C. rectus* (9.52%) were also detected in the samples evaluated in the present study (Table 6). Only a few studies have looked for different bacterial species in the same atheromatous plaques[23, 29] and while Zaremba et al.[29] were able to detect DNA from all targeted bacteria, Padilla et al.[23] did not detect any of specific DNA in their studied samples (Table 6). This heterogeneity in the results may be attributed to differences in the methodology, including atheromatous plaque collection, homogenization, DNA extraction and the PCR technology used.

In this investigation we studied carotid arteries obtained by endarterectomy. Other reports have studied atheromatous plaques from coronary arteries[24, 27, 29, 34, 37], carotid arteries[23, 25, 26, 36, 41, 42], aortas[35, 38, 39], mitral valve specimens, aortic aneurysmal wall specimens[40], saphenous veins or mammary arteries[28]. Although endarterectomy has been the most frequently reported procurement procedure[23, 25-27, 41, 42], other investigations have collected samples from patients scheduled for coronary artery bypass graft[28,24,29,37]. The amount of atheromatous plaque specimen has also varied among the studies. Some authors have used approximately 100 mg from the inner part of the atheromatous samples[25, 27, 34, 37, 42], whilst others used a midsection[26], or did not specify it at all[28, 38, 40]. The process of homogenization of the sample was usually not reported[23, 24, 27, 29, 37, 38, 40-42], although when this process was detailed, the authors utilized a mechanical homogenizer, as we have done in this investigation[25, 26, 28, 34-36]. The DNA was usually extracted using commercial kits[27, 34, 35, 37, 38, 41], although some studies also used the phenol-chloroform-isoamylic alcohol purification method[25, 26, 28]. In this investigation, we optimised this method by adding a final step with phenol:chloroform:isoamyl alcohol (1:4:24).

For detecting bacterial DNA, the majority of authors have used PCR technology, utilising either Real-Time PCR[27], PCR with specific sets of primers[24, 34, 35, 37, 38, 40, 42] or nested PCR[26, 36]. Padilla et al.[23] made first a bacteriological culture of the homogenized samples, and then amplified with PCR the isolates. In this study, we have used nested PCR, as this method facilitates the detection of bacterial DNA present at very low levels[30]. By using a two-step PCR amplification procedure, there is a clear increase in the sensitivity of the process[43], although the decrease in specificity has to be considered. For the avoidance of cross-reactivity, we included several negative controls in each batch of experiments. Nested-PCR has been previously used for detecting periodontal pathogens in gingival crevicular fluid (GCF) samples[43], in atheromatous plaques[26, 36] or for detecting *Helicobacter pylori* in saliva and GCF samples[44, 45]. Different from the results reported in this study, Aimetti et al.[26] and Fiehn et al.[36] also studying atheromatous plaques from carotid arteries and using nested PCR did not detect DNA from almost any of the bacterial species. These differences are probably due to the different amplification methods used, since we optimised the PCR reaction by using first the broad-spectrum primers (first PCR) and by concentrating the amplicons obtained before carrying out the second step of the nested PCR (primers from specific bacteria)[31].

The main limitation of this study was our inability to retrieve the clinical information from all the patients, since only half of the patients provided complete

clinical data. The collected clinical data, however, allowed us to demonstrate a positive correlation between the patient's periodontal status with the prevalence of periodontal pathogens detected in their atheromatous plaques. The patients´s diagnosis was either edentulous or suffering moderate to advanced chronic periodontitis, since there were no healthy or gingivitis patients in this sample population. The prevalence of DNA from *A. actinomycetemcomitans* in atheromatous samples was significantly higher in dentate patients when compared with edentulous and its prevalence also showed a significant positive linear trend with the number of teeth present. Prevalence of DNA from *P. gingivalis* and *T. forsythia* was also higher in dentate versus non-dentate patients, although these differences were not statistically significant. These findings can be explained since the teeth may act as a reservoir of *P. gingivalis* or *A. actinomycetemcomitans* in the oral cavity [46, 47] and the presence of these bacteria in high numbers in the subgingival microbiota in close vicinity with the ulcerated epithelium at the biofilm-gingival interface, may explain the likely bacterial invasion through bacteremias and their translocation to other parts of the body[13, 15, 48]. Further indirect evidence of these events is the positive correlation between probing pocket distribution and bacterial DNA presence. *E. corrodens* and *F. nucleatum* were observed in significantly higher prevalence in patients with at least one pocket greater than 6 mm. A similar trend, although not statistically significant was observed for *P. gingivalis* or *A. actinomycetemcomitans*. These results, however, need to be interpreted with caution due to the limited sample size and since this population lacked dentate patients without pockets deeper than 4 mm. Furthermore, another limitation of this study was the lack of information on the composition of the subgingival microbiota from the same patients, what would have enabled us to correlate these pathogens with the bacterial DNA observed. We were unable to retrieve subgingival plaque samples from these patients at the time of the periodontal examinations, since all patients were prescribed systemic antibiotics after the vascular surgery and this would have altered the microbiological results. Other studies have also reported a positive correlation between species prevalent in dental plaque and their detection in cardiovascular specimens[23, 26, 28, 29, 34, 38, 40, 42].

All 22 patients who underwent the periodontal examination were either smokers or former smokers. As tobacco smoking is a common risk factor for both pathologies, this major confounder may explain in part the associations between periodontitis and CVD reported in this investigation[49].

Within the limitations of this investigation, we have identified periodontitis-associated bacterial DNA in carotid artery atheromatous plaques retrieved by endarterectomy. These findings provide additional evidence that supports the potential association between periodontitis and CVD, in which bacteria present in the subgingival biofilm gain access to the systemic circulation (bacteremia), colonize at distant sites and thus, they might influence the patho-physiology of atherogenesis[5, 6]. The mere presence of bacterial DNA in these atheromatous plaques, however, does not imply that live bacteria are present within the plaques and therefore, further investigations are warranted. These studies should seek microbiological data not only from the atheromatous plaques, but also from the gingival crevicular fluid and serum from the same patients, thus being able to confirm this likely direct relationship between periodontitis and CVD.

CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interests.

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Table 1: Specific primers used

Bacteria	Sequence (5´- 3´)	Positions	Length (bp)
A.actinomycetemcomitans	AAACCC ATCTCTGAG TTCTTCTTC	F478	557
	ATGCCA ACTTGA CGT TAA AT	R1034	
P. gingivalis	AGGCAG CTTGCC ATA CTGCG	F729	404
	ACT GTT AGCAACTAC CGATGT	R1132	
E. corrodens	CTAATACCGCATACGTCCTAAG	F169	688
	CTACTAAGCAATCAAGTTGCCC	R856	
C. rectus	TTTCGGAGCGTAAACTCCTTTTC	F415	598
	TTTCTGCAAGCAGACACTCTT	R1012	
T. forsythia	GCGTATGTAACCTGCCCGCA	F120	641
	TGCTTCAGTGTCAGTTATACCT	R760	
F. nucleatum	TAAAGCGCGTCTAGGTGGTT	F517	697
	ACGGCTTTGCAACTCTCTGT	R1214	

Table 2: Pure cultures of bacteria used as positive controls.

Microorganism	Collection	Reference number
P. gingivalis	ATCC	33277
A. actinomycetemcomitans	DSM	8324
T. forsythia	ATCC	43037
F. nucleatum	DSM	20482
E. corrodens	NCTC	10596
C. rectus	NCTC	11489

ATCC: American Type Culture Collection;

DSM: Deutsche Sanmlung von Mikrooganismen;

NCTC: National Collection of Type Cultures.

Table 3: Simultaneous detection of bacterial DNA in the same atheromatous plaque.

Bacteria	Prevalence
Aa and *Pg*	61.90% (26/42)
Pg and *Tf*	50.00% (21/42)
Aa and *Tf*	50.00% (21/42)
Pg, *Aa* and *Tf*	47.62% (20/42)
Pg, *Aa*, *Tf* and *Ec*	35.71% (15/42)
Pg, *Aa*, *Tf*, *Ec* and *Fn*	28.57% (12/42)

Table 4. Detection rates of periodontal bacteria in patients with different number of teeth (%).

	Pg	*Aa*	*Tf*	*Ec*	*Cr*	*Fn*
Edentulous (n=4)	50.0	0.0	25.0	50.0	50.0	50.0
Dentate (n=18)	88.9	83.3	72.2	61.1	0	44.4
P value	0.068	0.001	0.076	0.683	0.002	0.916
(1) 0-10 (n=7)	57.1	28.6	42.9	57.1	28.6	57.1
(2) 11-20 (n=10)	90.0	90.0	70.0	50.0	0	33.3
(3) 21-32 (n=5)	100.0	80.0	80.0	80.0	0	60.0
P value	0.052	0.040	0.179	0.495	0.072	0.978

(1) (2) (3) number of teeth present

Table 5. Detection rates of periodontal bacteria in patients with different probing pocket depth distribution (%).

	Pg	*Aa*	*Tf*	*Ec*	*Cr*	*Fn*
≤6mm (n=8)	75.0	87.5	50.0	25.0	0.0	14.3
>6mm (n=10)	100	80.0	90.0	90.0	0.0	70.0
P value	0.094	0.671	0.060	0.005	---	0.024

Table 6. Frequency of detection of different periodontal bacteria in vascular specimens published in the literature.

Author, year	Method	n	*Pg*	*Aa*	*Tf*	*Ec*	*Cr*	*Fn*
Present study	Nested PCR	42	78.6	66.7	61.9	54.8	9.5	52.4
Gaetti-Jardim 2009[27]	Real-time PCR	44	53.8	46.2	25.6	---	---	0
Nakano, 2009 [40]	Specific PCR	223*	20	30	---	---	5	---
Elkaim, 2008 [28]	Hybridization	22	54.5	54.5	---	---	---	54.5
Zhang, 2008 [37]	Specific PCR	51	33	0	31	---	---	12
Zaremba, 2007 [29]	Hybridation	20	50	5	25	20	15	15
Aimetti, 2007 [26]	Nested PCR	33	0	0	0	---	---	---
Romano, 2007 [25]	Hybridation	21	0	0	0	---	---	---
Pucar, 2007 [24]	Specific PCR	15	53.3	26.7	13.3	---	---	---
Padilla, 2006 [23]	Culture- PCR	12	0	16.7	0	0	0	0
Marques da Silva, 2005 [35]	Specific PCR	51	0	7.1	0	---	---	---
Fiehn, 2005 [36]	Nested PCR	24	4.2	0	0	---	0	---
Cairo, 2004 [42]	Specific PCR	52	0	0	0	---	---	0
Ishishara, 2004 [34]	Specific PCR	51	21.6	23.3	5.9	---	---	---
Kurihara, 2004 [38]	Specific PCR	32	85	0	22	---	45	---
Stelzel, 2002 [39]	Specific PCR	26	15.4	0		---	---	---
Haraszthy, 2000 [41]	Specific PCR	50	26	18	30	---	---	---

¶ G-Nome DNA kit. MP Biomedical S. LLC Solon, Ohio, USA

IKA. Weke GMBH & CO.KG, Stanfen, Germany

** W4502, Sigma-Aldrich Quimica SA, Madrid, Spain

†† illustra™, GE Healhcare, Buckinghamshire, United Kingdom

‡‡ Agarose D-2. Pronadisa, CONDA, Torrejón de Ardoz, Madrid, Spain

§§ Gel Printer Plus, TD, Alcobendas, Madrid, Spain

‖‖ Invitrogen, Carisbad, USA

¶¶ Hu-Friedy, Leimen, Germany

SPSS for Windows (SPSS Inc. version 17.0)