

# Detection of Periodontal Bacteria in Atheromatous Plaque by Nested Polymerase Chain Reaction

Elena Figuero,\*† María Sánchez-Beltrán,† Susana Cuesta-Frechoso,‡ Jose María Tejerina,‡ Jose Antonio del Castro,§ Jose María Gutiérrez,§ David Herrera,\*|| and Mariano Sanz\*||

**Background:** In recent years, increasing evidence regarding the potential association between periodontal diseases and cardiovascular diseases has been identified. The available evidence underlines the importance of detecting periodontal pathogens on atheromatous plaque as the first step in demonstrating the causal relationship between the two conditions. The main aim of this investigation is to detect periodontitis-associated bacteria from carotid artery atheromatous plaque from patients who received an endarterectomy using strict sample procurement and laboratory procedures.

**Methods:** Atheromatous plaque from endarterectomies from carotid arteries were scraped and homogenized, and bacterial DNA was extracted. To obtain a representative concentration of amplicons, two amplifications of the bacterial 16S ribosomal-RNA gene were carried out for each sample with universal eubacteria primers by a polymerase chain reaction (PCR). A nested PCR with specific primers for the target bacteria was performed next. Statistical tests included the  $\chi^2$  test.

**Results:** Forty-two atheromatous plaque were analyzed. All of them were positive for  $\geq 1$  target bacterial species. The bacterial species most commonly found was *Porphyromonas gingivalis* (78.57%; 33 of 42), followed by *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*) (66.67%; 28 of 42), *Tannerella forsythia* (previously *T. forsythensis*) (61.90%; 26 of 42), *Eikenella corrodens* (54.76%; 23 of 42), *Fusobacterium nucleatum* (50.00%; 21 of 42), and *Campylobacter rectus* (9.52%; four of 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 plaque retrieved by endarterectomy is confirmed. *J Periodontol* 2011;82:1469-1477.

## KEY WORDS

Atherosclerosis; cardiovascular diseases; microbiology; periodontal diseases; polymerase chain reaction; *Porphyromonas gingivalis*.

\* Section of Graduate Periodontology, Faculty of Dentistry, Complutense University, Madrid, Spain.

† Research Laboratory, Complutense University.

‡ Faculty of Dentistry, University of Oviedo, Asturias, Spain.

§ Department of Angiology and Vascular Surgery, University Central Hospital Asturias, Oviedo, Spain.

|| Etiology and Therapy of Periodontal Disease Research Group, Faculty of Dentistry, Complutense University.

**A**therosclerosis is the major event in the pathophysiology of cardiovascular diseases (CVDs), in which large- to medium-size muscular and large elastic arteries become occluded with fibrolipidic lesions, known as atheromas. These atheromatous plaque are responsible for end-stage complications or events associated with CVDs, such as coronary thrombosis, acute myocardial infarction, and stroke.<sup>1</sup>

In recent years, increasing evidence regarding the potential association between periodontal diseases and CVDs was identified.<sup>1-6</sup> Meta-analyses<sup>2-4</sup> showed that this association was statistically significant and consistent, although of a low magnitude. These studies<sup>2-4</sup> supported the hypothesis that periodontitis may confer an independent risk to CVDs. Because of the high prevalence of periodontitis in humans, and because CVDs are the main cause of death in developed countries, an increasing interest was raised in the scientific community to identify the potential links between both entities.<sup>5,6</sup>

Some hypotheses were proposed to explain why periodontitis may increase the risk of CVDs,<sup>6,7</sup> from an indirect association determined by common risk factors or a common phenotype underlining both conditions<sup>8</sup> to a direct association between the periodontal infection and the pathophysiology of the atherosclerotic lesion.<sup>9</sup> This last hypothesis was 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.<sup>5</sup> However, a possible common predisposition underlying both diseases might coexist with the direct influence of periodontitis to CVDs.<sup>6</sup> Periodontitis is a chronic inflammatory disease of multifactorial etiology, where the primary etiological factor is the presence of specific bacteria residing in the subgingival biofilm. This subgingival biofilm is a complex microbiota where >700 bacterial species were detected;<sup>10</sup> however, only a limited number of these bacteria were shown to be a risk factor for the initiation or progression of periodontitis, namely *Aggregatibacter actinomycetemcomitans* (*Aa*) (previously *Actinobacillus actinomycetemcomitans*), *Porphyromonas gingivalis* (*Pg*), and *Tannerella forsythia* (*Tf*) (previously *T. forsythensis*).<sup>11</sup> The presence of these periodontal pathogens was demonstrated to be associated with diseased sites and healthy sites, albeit in low numbers. Frequency of detection was also shown to vary in different geographical locations (e.g., the prevalence of *Pg* was higher in Spain than in other countries, such as The Netherlands).<sup>12</sup>

These periodontal pathogens present in the subgingival biofilm were also identified in the blood of patients with periodontitis, mostly associated with periodontal interventions, although also after normal day-life activities such as mastication,<sup>13</sup> toothbrush-

ing,<sup>14</sup> or dental flossing.<sup>15</sup> The occurrence of these bacteremias raised the hypothesis of bacterial colonization at distant sites and their specific involvement in the pathogenesis of the atherosclerotic lesion. Experimental studies<sup>16-21</sup> showed the potential ability of these pathogens being involved in different stages of the development of the atherosclerosis lesion, mainly by: 1) favoring the adherence of leukocytes to the vascular endothelium by increasing the expression of vascular cellular adhesion molecule-I, intracellular adhesion molecule-I, and E-selectin in human aortic endothelial cells;<sup>16</sup> 2) favoring the migration of monocytes through the expression of monocyte chemoattractant protein-I in endothelial cells infected with *P. gingivalis*;<sup>17</sup> 3) promoting the transformation of macrophages into foam cells;<sup>18,19</sup> 4) having a procoagulant effect;<sup>20</sup> and 5) favoring the rupture of the atheromatous plaque through the release of metalloproteinases.<sup>21</sup> This experimental evidence, although in vitro, underlined the importance of detecting and identifying these putative pathogens on atheromatous plaque as the first step in understanding the possible associations between periodontitis and CVDs.

The identification of periodontal bacteria DNA in atheromatous plaques was first reported in 1999 from samples of human carotid endarterectomies.<sup>22</sup> Since then, other similar investigations<sup>23-29</sup> have reported conflicting results (Table 6), which may be attributed to differences in the procurement of specimens or to differences in the molecular technique used for bacterial DNA identification. The expected amount of bacteria is small, and because atheromatous plaque are complex lesions to extract DNA, there is a need for strict laboratory protocols aimed for sensitive and specific detection of bacterial DNA.<sup>30,31</sup> Therefore, the aim of this investigation is to detect DNA from periodontitis-associated bacteria in carotid-artery atheromatous plaque recovered from patients who received an endarterectomy using strict sample-procurement and laboratory procedures. Our hypothesis was that bacterial DNA from periodontopathic bacteria would be present in the retrieved atherosclerosis samples, and this presence would be related to the oral health status of the patients.

## MATERIALS AND METHODS

### Sample

The study sample consisted of atheromatous plaque retrieved during endarterectomy surgical procedures from the carotid artery of consecutive patients admitted to the Department of Angiology and Vascular Surgery, University Central Hospital Asturias (HUCA) because of various manifestations of CVD (symptomatic patients with stenosis <70% or asymptomatic patients with preocclusive stenosis). The inclusion period was December 2006 to January 2008. Patients

who fulfilled inclusion criteria were informed of the scope of the study and provided written informed consent previously approved by the ethics committee, HUCA. 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  $-20^{\circ}\text{C}$ , and sent to the Research Laboratory, Faculty of Dentistry, Complutense University, Madrid, Spain by a special courier service that maintained the  $-20^{\circ}\text{C}$  temperature until processing.

### Specimen Homogenization

Transport vials were opened at the research laboratory, taking care to maintain the aseptic handling of the specimens. The inner part of the plaque was scraped with a sterile blade, weighed, and transferred to 15-mL sterile plastic tubes with suspension buffer.<sup>¶</sup> Specimen homogenization was achieved with a mechanical homogenizer<sup>#</sup> until a uniform suspension was obtained.

### DNA Extraction

Total bacterial DNA from the whole homogenized samples was extracted by using a commercial kit.<sup>\*\*</sup> 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 milliliters phenol:chloroform:isoamyl alcohol solution were added and centrifuged at  $9,000 \times g$  for 15 minutes. The resulting supernatant was used, and 2 mL Tris-EDTA buffer was carefully added to the mixture. DNA was precipitated with ice-cold pure ethanol and resuspended in 50  $\mu\text{L}$  water.<sup>††</sup>

### Polymerase Chain Reaction (PCR) Amplifications

An amplification of the bacterial 16S ribosomal-RNA gene 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 U Taq DNA polymerase, 1 $\times$  polymerase buffer with 2 mM  $\text{MgCl}_2$ , 0.2 mM of a mixture of each deoxynucleoside triphosphate, 1  $\mu\text{M}$  primers, and 10  $\mu\text{L}$  template DNA in a total volume of 50  $\mu\text{L}$ . Samples were preheated at  $95^{\circ}\text{C}$  for 2 minutes followed by an amplification under the following conditions: denaturation at  $94^{\circ}\text{C}$  for 30 seconds, annealing at  $55^{\circ}\text{C}$  for 1 minute, and elongation at  $72^{\circ}\text{C}$  for 1 minute. Thirty-five cycles were performed followed by an elongation step at  $72^{\circ}\text{C}$  for 10 minutes.

Duplicate reactions were prepared for each sample. Obtained PCR products were purified and concentrated with a purification kit<sup>‡‡</sup> in a unique final solution.

Second, a nested PCR with specific primers for periodontal pathogens (*Aa*, *Pg*, *Eikenella corrodens* [*Ec*], *Campylobacter rectus* [*Cr*], *Tf*, and *Fusobacterium nucleatum* [*Fn*]) was performed. Primers were designed on the basis of the 16S ribosomal-RNA gene reported by Ashimoto et al.<sup>32</sup> (Table 1).

PCR amplification was performed in a master-mix solution containing 0.2 U Taq DNA polymerase, 1 $\times$  polymerase buffer with 2 mM  $\text{MgCl}_2$ , 0.2 mM of a mixture of each deoxynucleoside triphosphate, 1  $\mu\text{M}$  primers, and 2  $\mu\text{L}$  PCR products as template DNA in a total volume of 25  $\mu\text{L}$ . 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  $10^9$  to  $10^1$  colony forming units (CFU/mL). Irrespective of the pathogen, the methodology allowed for the amplification of  $10^2$  CFU/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- $\mu\text{L}$  aliquot of amplified sample from each PCR was electrophoresed through a 1% agarose gel<sup>§§</sup> in Tris-acetate EDTA buffer. The gel was stained with ethidium bromide (10 mg/mL) and visualized under an ultraviolet light transilluminator.<sup>|||</sup> A DNA ladder<sup>¶¶</sup> was used as molecular weight marker. The band position of PCR products was in accordance with the length of primers.

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

¶ G-Nome DNA kit, MP Biomedicals, Solon, OH.

# IKA-Werke GmbH & Co. KG, Staufen, Germany.

\*\* G-Nome DNA kit, MP Biomedicals.

†† W4502, Sigma-Aldrich Quimica, Madrid, Spain

‡‡ Illustra, GE Healthcare, Little Chalfont, United Kingdom.

§§ Agarose D-2, Pronadisa, CONDA, Madrid, Spain.

||| Gel Printer Plus, Technology for Diagnosis and Investigation, Madrid, Spain.

¶¶ Invitrogen, Carlsbad, CA.

**Table 1.**  
**Specific Primers Used**

Bacteria	Sequence (5'-3')	Positions	Length (bp)
<i>Aa</i>	AAACCCATCTCTGAGTTCTTCTTC ATGCCAACTTGACGTTAAAT	F478 R1034	557
<i>Pg</i>	AGGCAGCTTGCCATACTGCG ACTGTTAGCAACTACCGATGT	F729 R1132	404
<i>Ec</i>	CTAATACCGCATACTGCTTAAG CTACTAAGCAATCAAGTTGCC	F169 R856	688
<i>Cr</i>	TTTCGGAGCGTAACTCCTTTTC TTTCTGCAAGCAGACTCTT	F415 R1012	598
<i>Tf</i>	GCGTATGTAACTGCCCGCA TGCTTCAGTGTCAGTTATACCT	F120 R760	641
<i>Fn</i>	TAAAGCGCTCTAGGTGGTT ACGGCTTTGCAACTCTCTGT	F517 R1214	697

bp = base pairs.

**Table 2.**  
**Pure Cultures of Bacteria Used as Positive Controls**

Bacteria	Collection	Reference Number
<i>Pg</i>	ATCC	33,277
<i>Aa</i>	DSMZ	8,324
<i>Tf</i>	ATCC	43,037
<i>Fn</i>	DSMZ	20,482
<i>Ec</i>	NCTC	10,596
<i>Cr</i>	NCTC	11,489

ATCC = American Type Culture Collection, Barcelona, Spain; DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; NCTC = National Collection of Type Cultures, Salisbury, UK.

**Periodontal Examination**

Once recovered from endarterectomies, all patients were invited for a consultation to obtain demographic information, smoking habits, and dental and medical histories. Patients underwent a complete oral examination by a single trained periodontist (JMT) who collected data on tooth loss, pocket probing depths (PDs), and mobility (grade I, II or III) at six sites per tooth, excluding third molars, using a periodontal probe.<sup>##</sup> Patients also had a panoramic radiograph taken, where the percentage of bone loss was calculated. Based on clinical and radiographic data, patients were diagnosed with periodontitis or as healthy/having gingivitis.<sup>33</sup>

**Data Analyses**

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

The frequency of pathogen detection in atheromatous plaque was obtained for each patient. In addition, patients with periodontal data were stratified according to their number of teeth (edentulous, zero to 10, 11 to 20, and >20 teeth) and to the proportion of PDs (1 to 3 mm, shallow; 4 to 6 mm, intermediate; and >6 mm, deep). Differences in terms of the prevalence of pathogens in these subgroups were determined by the  $\chi^2$  test. Statistical significance was established at the 95% confidence level. A statistical software package<sup>\*\*\*</sup> was used for all data analyses.

**RESULTS**

**Data From Atheromatous Plaque**

A total of 42 atheromatous plaque were analyzed. The mean weight of the inner parts of atheromatous plaque was 201.68 mg (SD: 231.00 mg). All analyzed samples were positive for  $\geq 1$  target bacterial species. The bacterium most commonly found in atheromatous plaque was *Pg* (78.57%; 33 of 42), followed by *Aa* (66.67%; 28 of 42), *Tf* (61.90%; 26 of 42), *Ec* (54.76%, 23 of 42), *Fn* (50.00%, 21 of 42), and *Cr* (9.52%, four of 42).

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

**Patient-Based Data**

From the 42 patients who underwent vascular surgery, 22 patients (17 males and 5 females, aged 57 to 81 years; mean age: 71.1 years) agreed to participate in the oral health consultation, in which demographic and oral health information was obtained.

Half of the patients were surgically treated for a pre-occlusive asymptomatic stenosis, and the other half were surgically treated for a stenosis >70% symptomatic. Twelve patients were current smokers (10 to 60 cigarettes/day), 10 patients were former smokers; eight patients had hypertension, and six patients had diabetes.

<sup>##</sup> CPC-12, Hu-Friedy, Leimen, Germany.

<sup>\*\*\*</sup> SPSS for Windows, v.17.0, IBM, Chicago, IL.

Of the 22 patients who underwent periodontal examination, four patients were edentulous, and therefore, excluded from the intraoral data analysis. Patients reported tooth mobility as the main cause for tooth extraction; however, none had received previous periodontal treatment. The mean number of teeth present in the 18 dentate patients was 13.05 teeth (SD = 9.13 teeth) and the mean PD was 4.47 mm (SD = 0.83 mm). The proportions of sites with PDs <4, 4 to 6, or >6 mm were 17.87% (SD = 20.25%), 77.85% (SD = 18.80%), and 4.28% (SD = 7.69%), respectively. The proportion of teeth with mobility grade I was 49.25% (SD = 28.76%), with mobility grade II was 28.65% (SD = 21.75%), and with mobility grade III was 10.85% (SD = 16.54%). The average of bone loss measured in panoramic radiographs was 56%. The diagnosis of patients was either edentulous or with moderate to advanced chronic periodontitis.

**Presence of Periodontal Bacteria in Atheromatous Plaque Stratified by Patients Groups**

Dentate patients had significantly higher levels of *Aa* in atheromatous plaque than edentulous patients.

**Table 3.**  
**Simultaneous Detection of Bacterial DNA in the Same Atheromatous Plaque**

Bacteria	Prevalence (% [number of plaque])
<i>Aa</i> and <i>Pg</i>	61.90 (26 of 42)
<i>Pg</i> and <i>Tf</i>	50.00 (21 of 42)
<i>Aa</i> and <i>Tf</i>	50.00 (21 of 42)
<i>Pg</i> , <i>Aa</i> , and <i>Tf</i>	47.62 (20 of 42)
<i>Pg</i> , <i>Aa</i> , <i>Tf</i> , and <i>Ec</i>	35.71 (15 of 42)
<i>Pg</i> , <i>Aa</i> , <i>Tf</i> , <i>Ec</i> , and <i>Fn</i>	28.57 (12 of 42)

**Table 4.**  
**Percent of Periodontal Bacteria in Patients by Number of Teeth**

Subgroups	<i>Pg</i>	<i>Aa</i>	<i>Tf</i>	<i>Ec</i>	<i>Cr</i>	<i>Fn</i>
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
<i>P</i> value	0.068	0.001	0.076	0.683	0.002	0.916
0 to 10 teeth (n = 7)	57.1	28.6	42.9	57.1	28.6	57.1
11 to 20 teeth (n = 10)	90.0	90.0	70.0	50.0	0	33.3
21 to 32 teeth (n = 5)	100.0	80.0	80.0	80.0	0	60.0
<i>P</i> value	0.052	0.040	0.179	0.495	0.072	0.978

Dentate patients also had a higher prevalence of *Pg*, *Tf*, and *Ec*, although these differences were not statistically significant. By contrast, edentulous patients had higher levels of *Cr* and *Fn* (Table 4).

When patients were stratified by the number of teeth, a positive linear trend with the prevalence of *Aa* and *Pg* was observed (Table 4).

When the prevalence of bacterial species in atheromatous plaque was correlated with the PD distribution of patients, it was observed that patients with PDs >6 mm presented *Ec* and *Fn* more frequently than did patients with PDs ≤6 mm. A clear trend was also seen for *Pg* and *Tf*, although differences were not statistically significant (Table 5).

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

**DISCUSSION**

The present study aims to investigate the presence of DNA from periodontal bacteria in atheromatous plaque retrieved from patients who received endarterectomies because of various manifestations of ischemic vascular disease. These samples were subjected to meticulous laboratory procedures to optimize the sensitivity and specificity of detection. All analyzed samples were positive for the DNA of ≥1 target bacterial species. The DNA from bacteria most commonly found were *Pg* (78.57%) followed by *Aa* (66.67%) (Table 6). The concomitant detection of DNA from *Pg* and *Aa* was observed in 61.90% of samples. These results were similar to those found by Gaetti-Jardim et al.,<sup>27</sup> with 64.1% of clinical samples from periodontitis patients in which DNA from ≥2 species were detected.

Other authors<sup>24,27,29,34</sup> identified *Pg* and *Aa* as the most prevalent DNA from bacteria in atheromatous plaque. By contrast, some reports were not able to

**Table 5.**  
**Percent of Periodontal Bacteria in Patients With Different PD Distributions**

PD (mm)	<i>Pg</i>	<i>Aa</i>	<i>Tf</i>	<i>Ec</i>	<i>Cr</i>	<i>Fn</i>
≤6 (n = 8)	75.0	87.5	50.0	25.0	0.0	14.3
>6 (n = 10)	100	80.0	90.0	90.0	0.0	70.0
P value	0.094	0.671	0.060	0.005	1.0	0.024

detect *Pg*,<sup>23,35</sup> *Aa*,<sup>36-39</sup> or neither of the two<sup>25,26,40</sup> (Table 6).

DNA from *Tf* (61.90%), *Ec* (54.76%), *Fn* (50%), and *Cr* (9.52%) were also detected in the samples evaluated in the present study (Table 6). Only two studies<sup>23,29</sup> looked for different bacterial species in the same atheromatous plaque, and although Zaremba et al.<sup>29</sup> detected DNA from all targeted bacteria, Padilla et al.<sup>23</sup> did not detect any specific DNA in their studied samples (Table 6). This heterogeneity in the results may have been attributed to differences in the methodology, including atheromatous plaque collection, homogenization, DNA extraction, and PCR technology used.

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

For detecting bacterial DNA, the majority of authors used PCR technology, using either real-time PCR,<sup>27</sup> PCR with specific sets of primers,<sup>24,34,35,37,38,40,42</sup>

or nested PCR.<sup>26,36</sup> Padilla et al.<sup>23</sup> first made a bacteriologic culture of homogenized samples, and then amplified the isolates with PCR. In this study, we use a nested PCR because this method facilitated the detection of bacterial DNA present at very low levels.<sup>30</sup> The use of a two-step PCR amplification procedure results in a clear increase in the sensitivity of the process,<sup>43</sup> 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 was previously used for detecting periodontal pathogens in gingival crevicular fluid (GCF) samples<sup>43</sup> and atheromatous plaque<sup>26,36</sup> or for detecting *Helicobacter pylori* in saliva and GCF samples.<sup>44,45</sup> Different from the results reported in this study, Aimetti et al.<sup>26</sup> and Fiehn et al.,<sup>36</sup> who also studied atheromatous plaque from carotid arteries and used a nested PCR, did not detect DNA from almost any of the bacterial species. These differences were probably due to the different amplification methods used, because we optimized the PCR by first using 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).<sup>31</sup>

The main limitation of this study was our inability to retrieve the clinical information from all the patients because only half the patients provided complete clinical data. However, the collected clinical data allowed us to demonstrate a positive correlation between the patient's periodontal status with the prevalence of periodontal pathogens detected in their atheromatous plaque. The patient diagnosis was either edentulous or with moderate to advanced chronic periodontitis because there were no healthy or gingivitis patients in this sample population. The prevalence of DNA from *Aa* in atheromatous samples was significantly higher in dentate patients compared to edentulous patients, and its prevalence showed a significant positive linear trend with the number of teeth present. The prevalence of DNA from *Pg* and *Tf* was also higher in dentate versus non-dentate patients, although these differences were not statistically significant. These findings were explained because the teeth may act as a reservoir of *Pg* or *Aa* in the oral cavity,<sup>46,47</sup> 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 might have explained the likely bacterial invasion through bacteremias and their translocation to other parts of the body.<sup>13,15,48</sup> Further indirect evidence of these events was the positive correlation between the probing pocket distribution and bacterial DNA presence. *Ec* and *Fn* were observed in a significantly higher prevalence in patients with ≥1 pocket >6 mm deep.

**Table 6.**  
**Percent of Different Periodontal Bacteria in Vascular Specimens**

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

– = not determined.

A similar trend, although not statistically significant, was observed for *Pg* or *Aa*. However, these results needed to be interpreted with caution because of the limited sample size, and, since this population lacked dentate patients without pockets >4 mm deep. Furthermore, another limitation of this study was the lack of information on the composition of the subgingival microbiota from the same patients, which 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 periodontal examinations because all patients were prescribed systemic antibiotics after the vascular surgery, and this would have altered the microbiologic results. Other studies<sup>23,26,28,29,34,38,40,42</sup> also reported a positive correlation among species prevalent in dental plaque and their detection in cardiovascular specimens.

All 22 patients who underwent the periodontal examination were either smokers or former smokers. Because tobacco smoking is a common risk factor for both pathologies, this major confounder might have explained, in part, the associations between periodontitis and CVDs reported in this investigation.<sup>49</sup>

## CONCLUSIONS

Within the limitations of this investigation, we have identified periodontitis-associated bacterial DNA in carotid artery atheromatous plaque retrieved by endarterectomy. These findings provide additional evidence that supports the potential association between periodontitis and CVDs, in which bacteria present in the subgingival biofilm gain access to the systemic circulation (bacteremia), colonize at distant sites, and thus, might influence the pathophysiology of atherogenesis.<sup>5,6</sup> However, the mere presence of bacterial DNA in these atheromatous plaque did not imply that live bacteria were present within the plaque, and therefore, further investigations are warranted. These studies should seek microbiologic data from atheromatous plaque and GCF and serum from the same patients, thus being able to confirm this likely direct relationship between periodontitis and CVDs.

## ACKNOWLEDGMENT

The authors report no conflicts of interest related to this study.

## REFERENCES

- Paquette DW, Brodala N, Nichols TC. Cardiovascular disease, inflammation, and periodontal infection. *Periodontol 2000* 2007;44:113-126.
- Humphrey LL, Fu R, Buckley DI, Freeman M, Helfand M. Periodontal disease and coronary heart disease incidence: A systematic review and meta-analysis. *J Gen Intern Med* 2008;23:2079-2086.
- Bahekar AA, Singh S, Saha S, Molnar J, Arora R. The prevalence and incidence of coronary heart disease is significantly increased in periodontitis: A meta-analysis. *Am Heart J* 2007;154:830-837.
- Blaizot A, Vergnes JN, Nuwwareh S, Amar J, Sixou M. Periodontal diseases and cardiovascular events: Meta-analysis of observational studies. *Int Dent J* 2009;59:197-209.
- Sanz M, D'Aiuto F, Deanfield J, Fernández-Avilés F. European workshop in periodontal health and cardiovascular disease – Scientific evidence on the association between periodontal and cardiovascular diseases: A review of the literature. *Eur Heart J Suppl* 2010;12(Suppl. B):B3-B12.
- Bouchard P, Boutouyrie P, D'Aiuto F, et al. European workshop in periodontal health and cardiovascular disease consensus document. *Eur Heart J Suppl* 2010;12(Suppl. B):B13-B22.
- Chun YH, Chun KR, Olguin D, Wang HL. Biological foundation for periodontitis as a potential risk factor for atherosclerosis. *J Periodontol* 2005;40:87-95.
- Schaefer AS, Richter GM, Groessner-Schreiber B, et al. Identification of a shared genetic susceptibility locus for coronary heart disease and periodontitis. *PLoS Genet* 2009;5:e1000378.
- Li L, Messas E, Batista EL Jr., Levine RA, Amar S. *Porphyromonas gingivalis* infection accelerates the progression of atherosclerosis in a heterozygous apo-lipoprotein E-deficient murine model. *Circulation* 2002;105:861-867.
- Paster BJ, Boches SK, Galvin JL, et al. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;183:3770-3783.
- Consensus report. Periodontal diseases: Pathogenesis and microbial factors. *Ann Periodontol* 1996;1:926-932.
- Sanz M, van Winkelhoff AJ, Herrera D, Dellempijn-Kippuw N, Simón R, Winkel E. Differences in the composition of the subgingival microbiota of two periodontitis populations of different geographical origin. A comparison between Spain and The Netherlands. *Eur J Oral Sci* 2000;108:383-392.
- Förner L, Larsen T, Kilian M, Holmstrup P. Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation. *J Clin Periodontol* 2006;33:401-407.
- Hartzell JD, Torres D, Kim P, Wortmann G. Incidence of bacteremia after routine tooth brushing. *Am J Med Sci* 2005;329:178-180.
- Crasta K, Daly CG, Mitchell D, Curtis B, Stewart D, Heitz-Mayfield LJ. Bacteraemia due to dental flossing. *J Clin Periodontol* 2009;36:323-332.
- Roth GA, Moser B, Roth-Walter F, et al. Infection with a periodontal pathogen increases mononuclear cell adhesion to human aortic endothelial cells. *Atherosclerosis* 2007;190:271-281.
- Choi EK, Park SA, Oh WM, et al. Mechanisms of *Porphyromonas gingivalis*-induced monocyte chemoattractant protein-1 expression in endothelial cells. *FEMS Immunol Med Microbiol* 2005;44:51-58.
- Miyakawa H, Honma K, Qi M, Kuramitsu HK. Interaction of *Porphyromonas gingivalis* with low-density lipoproteins: Implications for a role for periodontitis in atherosclerosis. *J Periodontol Res* 2004;39:1-9.
- Giacona MB, Papapanou PN, Lamster IB, et al. *Porphyromonas gingivalis* induces its uptake by human macrophages and promotes foam cell formation in vitro. *FEMS Microbiol Lett* 2004;241:95-101.
- Nicu EA, Van der Velden U, Nieuwland R, Everts V, Loos BG. Elevated platelet and leukocyte response to oral bacteria in periodontitis. *J Thromb Haemost* 2009;7:162-170.
- Tuomainen AM, Jauhiainen M, Kovanen PT, Metso J, Paju S, Pussinen PJ. *Aggregatibacter actinomycetemcomitans* induces MMP-9 expression and proatherogenic lipoprotein profile in apoE-deficient mice. *Microb Pathog* 2008;44:111-117.
- Chiu B. Multiple infections in carotid atherosclerotic plaques. *Am Heart J* 1999;138:S534-S536.
- Padilla C, Lobos O, Hubert E, et al. Periodontal pathogens in atheromatous plaques isolated from patients with chronic periodontitis. *J Periodontol Res* 2006;41:350-353.
- Pucar A, Milasin J, Lekovic V, et al. Correlation between atherosclerosis and periodontal putative pathogenic bacterial infections in coronary and internal mammary arteries. *J Periodontol* 2007;78:677-682.
- Romano F, Barbui A, Aimetti M. Periodontal pathogens in periodontal pockets and in carotid atheromatous plaques. *Minerva Stomatol* 2007;56:169-179.
- Aimetti M, Romano F, Nessi F. Microbiologic analysis of periodontal pockets and carotid atheromatous plaques in advanced chronic periodontitis patients. *J Periodontol* 2007;78:1718-1723.
- Gaetti-Jardim E Jr., Marcelino SL, Feitosa AC, Romito GA, Avila-Campos MJ. Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries. *J Med Microbiol* 2009;58:1568-1575.
- Elkaïm R, Dahan M, Kocgozlu L, et al. Prevalence of periodontal pathogens in subgingival lesions, atherosclerotic plaques and healthy blood vessels: A preliminary study. *J Periodontol Res* 2008;43:224-231.
- Zaremba M, Górska R, Suwalski P, Kowalski J. Evaluation of the incidence of periodontitis-associated bacteria in the atherosclerotic plaque of coronary blood vessels. *J Periodontol* 2007;78:322-327.
- Apfalter P, Assadian O, Blasi F, et al. Reliability of nested PCR for detection of *Chlamydia pneumoniae* DNA in atheromas: Results from a multicenter study applying standardized protocols. *J Clin Microbiol* 2002;40:4428-4434.
- Clement B, Kehl L, DeBord K, Kitts C. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *J Microbiol Methods* 1998;31:135-142.
- Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;11:266-273.
- Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;4:1-6.

34. Ishihara K, Nabuchi A, Ito R, Miyachi K, Kuramitsu HK, Okuda K. Correlation between detection rates of periodontopathic bacterial DNA in coronary stenotic artery plaque [corrected] and in dental plaque samples. *J Clin Microbiol* 2004;42:1313-1315.
35. Marques da Silva R, Caugant DA, Lingaas PS, Geiran O, Tronstad L, Olsen I. Detection of *Actinobacillus actinomycesetemcomitans* but not bacteria of the red complex in aortic aneurysms by multiplex polymerase chain reaction. *J Periodontol* 2005;76:590-594.
36. Fiehn NE, Larsen T, Christiansen N, Holmstrup P, Schroeder TV. Identification of periodontal pathogens in atherosclerotic vessels. *J Periodontol* 2005;76:731-736.
37. Zhang YM, Zhong LJ, Liang P, Liu H, Mu LT, Ai SK. Relationship between microorganisms in coronary atheromatous plaques and periodontal pathogenic bacteria. *Chin Med J (Engl)* 2008;121:1595-1597.
38. Kurihara N, Inoue Y, Iwai T, Umeda M, Huang Y, Ishikawa I. Detection and localization of periodontopathic bacteria in abdominal aortic aneurysms. *Eur J Vasc Endovasc Surg* 2004;28:553-558.
39. Stelzel M, Conrads G, Pankuweit S, et al. Detection of *Porphyromonas gingivalis* DNA in aortic tissue by PCR. *J Periodontol* 2002;73:868-870.
40. Cairo F, Gaeta C, Dorigo W, et al. Periodontal pathogens in atheromatous plaques. A controlled clinical and laboratory trial. *J Periodontol Res* 2004;39:442-446.
41. Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ. Identification of periodontal pathogens in atheromatous plaques. *J Periodontol* 2000;71:1554-1560.
42. Nakano K, Nemoto H, Nomura R, et al. Detection of oral bacteria in cardiovascular specimens. *Oral Microbiol Immunol* 2009;24:64-68.
43. Leys EJ, Griffen AL, Strong SJ, Fuerst PA. Detection and strain identification of *Actinobacillus actinomycesetemcomitans* by nested PCR. *J Clin Microbiol* 1994;32:1288-1294.
44. Song Q, Haller B, Schmid RM, Adler G, Bode G. *Helicobacter pylori* in dental plaque: A comparison of different PCR primer sets. *Dig Dis Sci* 1999;44:479-484.
45. Bürgers R, Schneider-Brachert W, Reischl U, et al. *Helicobacter pylori* in human oral cavity and stomach. *Eur J Oral Sci* 2008;116:297-304.
46. Danser MM, van Winkelhoff AJ, de Graaff J, Loos BG, van der Velden U. Short-term effect of full-mouth extraction on periodontal pathogens colonizing the oral mucous membranes. *J Clin Periodontol* 1994;21:484-489.
47. Van Assche N, Van Assche M, Pauwels M, Teughels W, Quirynen M. Do periodontopathogens disappear after full-mouth tooth extraction? *J Clin Periodontol* 2009;36:1043-1047.
48. Lafaurie GI, Mayorga-Fayad I, Torres MF, et al. Periodontopathic microorganisms in peripheral blood after scaling and root planing. *J Clin Periodontol* 2007;34:873-879.
49. Hujoel PP, Drangsholt M, Spiekerman C, DeRouen TA. Periodontitis-systemic disease associations in the presence of smoking – Causal or coincidental? *Periodontol 2000* 2002;30:51-60.

Correspondence: Dr. Elena Figuero, Research Laboratory, Complutense University, Madrid, Plaza Ramón y Cajal, SN, 28040, Madrid, Spain. Fax: 34-913941910; e-mail: efigueruiz@gmail.com.

Submitted November 28, 2010; accepted for publication February 2, 2011.