J Clin Periodontol 2011; 38: 418–427 doi: 10.1111/j.1600-051X.2011.01717.x

Journal of Clinical Periodontology

Detection of specific periodontal microorganisms from bacteraemia samples after periodontal therapy using molecular-based diagnostics

Diana Marcela Castillo[1], María Carmen Sánchez-Beltrán[2], Jaime Eduardo Castellanos[1], Ignacio Sanz[2], Isabel Mayorga-Fayad[1], Mariano Sanz[2] and Gloria Inés Lafaurie[1]

[1]Group UIBO (Oral Basic Research Unit), Faculty of Odontology, University of El Bosque, Bogota, Colombia; [2]Group ETEP, Faculty of Odontology, University Complutense of Madrid, Madrid, Spain

Castillo DM, Sánchez-Beltrán MC, Castellanos JE, Sanz I, Mayorga-Fayad I, Sanz M, Lafaurie GI. Detection of specific periodontal microorganisms from bacteraemia samples after periodontal therapy using molecular-based diagnostics. J Clin Periodontol 2011; 38: 418–427. doi: 10.1111/j.1600-051X.2011.01717.x.

Abstract

Aim: The aim of this study was to assess the presence of subgingival pathogens in peripheral blood samples from periodontitis patients before and after scaling and root planing (Sc/RP) using nested polymerase chain reaction (nested PCR).

Materials and Methods: Peripheral blood samples were obtained from 42 patients with severe generalized chronic or aggressive periodontitis. In each patient, four samples of peripheral blood were drawn at different times: immediately before the Sc/RP procedure; immediately after Sc/RP; 15 and 30 min. post-Sc/RP. Blood samples were analysed for bacteraemia with anaerobic culturing and nested PCR, using universal bacterial primers that target the 16S-rRNA gene of most bacteria, subsequently re-amplified with specific primers to *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Eikenella corrodens*, *Campylobacter rectus* and *Prevotella intermedia*, using a modified phenol–chloroform method for DNA extraction.

Results: Presence of specific periodontal pathogens in peripheral blood after treatment was detected in 54.8% of the patients, in 47.6% with anaerobic culturing and in 19% with nested PCR. In 16.6%, the periodontal pathogens were detected before Sc/RP. *P. gingivalis* and *A. actynomicetemcomitans* were the pathogens most frequently detected in the bloodstream before and after Sc/RP.

Conclusions: Nested PCR demonstrated the presence of DNA from periodontal pathogens in blood samples in severe periodontitis patients before, during and after periodontal therapy. The use of these molecular-based techniques may improve the accuracy from the results obtained by haemoculture.

Key words: *Aggregatibacter actinomycetemcomitans*; bacteraemia; cardiovascular disease; infective endocarditis; nested PCR; periodontal disease; polymerase chain reaction (PCR); *Porphyromonas gingivalis*; scaling and root planing

Accepted for publication 7 February 2011

Conflict of interest and source of funding statement

The authors declare that they do not have any conflict of interest in relation to this investigation.

The study was funded by the ETEP Research Group at the Universidad Complutense of Madrid, Spain and by the Institute of Science and Technology, Francisco José de Caldas (COLCIENCIAS Grant No 1308-04-11854) and the Division of Research of the Universidad El Bosque, Bogotá, Colombia.

The current paradigm on the aetiopathogenesis of periodontitis is based on the definition of periodontitis as a chronic inflammatory disease caused by bacteria residing in the subgingival biofilm. *Porphyromonas gingivalis*, *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* have been the specific pathogens most frequently associated with this disease, being present in high numbers at deepened periodontal pockets and in severe periodontal conditions (Dzink et al. 1988, Ali et al. 1996, Ashimoto et al.1996, Haffajee et al. 2008). In these patients, several studies have reported the identification of these periodontal pathogens in peripheral blood samples (bacteraemia), usually provoked by different stimuli, as in periodontal interventions in which the roots are mechanically debrided, such as with ultrasonic scaling (Forner

et al. 2006) and scaling and root planing (Messini et al. 1999, Lafaurie et al. 2007b). Presence of these pathogens in bacteraemia samples, although in lower frequency, has also been reported associated to oral hygiene practices, such as toothbrushing (Lockhart et al. 2008) and flossing (Castra et al. 2009). Presence of lipopolysaccharides from these causative bacteria has also been reported in periodontitis patients after chewing (Geerts et al. 2002, Ide et al. 2004), and indirect evidence of bacteria in peripheral blood has also been measured by assessing specific antibody titres against these putative periodontal pathogens. In fact, the presence of high levels of IgA and IgG to periodontal pathogens such as *P. gingivalis* or *A. actinomycetemcomitans* in serum, has been significantly associated to the risk of developing a cardiovascular event or developing a secondary cardiovascular event in a recent systematic review (Mustapha et al. 2007) suggesting that the level of systemic exposure to these pathogens may be a marker of cardiovascular risk in atherosclerosis-related diseases. Another indirect evidence of bacteraemia has been the identification of DNA from periodontal pathogens in atherosclerotic plaques (Elkaïm et al. 2008, Nakano et al. 2009).

For the direct assessment of periodontal pathogens in peripheral blood, haemoculture has been the diagnostic method mostly used (Messini et al. 1999, Forner et al. 2006, Lafaurie et al. 2007b). This technique, however, requires the use of enriched media, which makes its sensitivity unreliable and it is unable to detect low numbers of bacteria. These drawbacks have steered the attention towards diagnostic methods based on molecular techniques that may increase the sensitivity and specificity of detecting these putative periodontal pathogens, because their identification does not need the enhanced growth of the bacteria and a positive diagnosis would include the detection of dead organisms that may be present in the bloodstream after being degraded by the host response. The use of polymerase chain reaction (PCR) using universal bacterial primers to the 16S ribosomal RNA gene was used previously to assess bacteraemia in periodontitis patients (Kinane et al. 2005). There are, however, studies reporting the use of molecular-based diagnostics for detecting DNA from periodontal pathogens using specific primers in bacteraemia samples. The aim of this study was, therefore, to assess the presence of DNA from specific periodontal pathogens in peripheral blood samples from patients with periodontitis, before and after scaling and root planing (Sc/RP), using the nested polymerase chain reaction(nested PCR) assay and to compare these results with the previously reported haemoculture data from the same patients (Lafaurie et al. 2007b).

Materials and Methods

Study population

The study population and the clinical description of this sample have been reported previously (Lafaurie et al. 2007b). In brief, the sample included 42 voluntary patients, 27 with a diagnosis of generalized severe chronic periodontitis (GChP) and 15 with a diagnosis of generalized aggressive periodontitis (GAgP), selected from those attending the periodontal clinic at the University of El Bosque, Bogota, Colombia. These subjects were selected after being periodontally examined, including the assessment of probing pocket depths, clinical attachment levels and bleeding on probing and being diagnosed of severe periodontal destruction defined by the presence of at least 10 sites with probing depth $\geqslant$7 mm and being subsequently diagnosed as GChP or GAgP (Armitage 1999). Exclusion criteria were reported previously (Lafaurie et al. 2007b). Selected patients entered into the study after being informed of the objectives of the investigation and signing an informed consent approved by the Institutional Review Board of the University of El Bosque.

Clinical protocol and blood sampling procedures

In each of the selected patients, four samples of peripheral blood were drawn from the cubital vein following the protocol described previously in the first publication from this study (Lafaurie et al. 2007b). In short, blood samples were drawn at four different times: immediately before the Sc/RP procedure (T_1), immediately after the Sc/RP procedure (T_2), 15 min. (T_3) and 30 min. post-Sc/RP (T_4), In each sampling time, 10 ml of blood were drawn; 5 ml were collected in the haemoculture tube *Ruiz Castañeda* (Bio-Bacter®, Bogotá, Colombia) containing a bi-phasic medium [one solid phase (tipricase soy agar) and one liquid phase (tripticase soy broth, sodium polyanetholesulfonate and sucrose)] enriched with haemin (0.0005%) and menadione (0.00005%), for the selective growth of anaerobic bacteria. The other 5 ml were collected in sterile tubes containing sodium citrate (Vacutainer, BD Biosciences, Bergen County, NJ, USA) and processed for bacterial DNA extraction immediately after sampling. This purified DNA was stored at -80°C until being processed for nested PCR.

On the day of the blood sample collection, patients were requested not to brush their teeth before the appointment and to consume only liquids during breakfast. In order to standardize the periodontal intervention, Sc/RP was carried out by the same operator (G. L.) using 1 min. for each site, in each of the 10 selected sites for a total of 10 min./patient.

Subgingival plaque samples

In all patients, subgingival plaque samples were taken on the same day in deep periodontal sites with sterile paper points before carrying out the subgingival instrumentation and transported in VMGA III medium. The identification of periodontal pathogens from subgingival plaque samples was performed through standard culture and PCR methods. In brief, 100 μl sample aliquots of tenfold dilutions were plated in agar brucella blood medium enriched with haemin and menadione (BBL Microbiology Systems®, Cockeysville, MD, USA) and incubated at 36°C during 7 days in an anaerobic atmosphere (Anaerogen, Oxoid, Hampshire, UK). Undiluted sample aliquots were plated in tripticase soy bacitracinevancomicine (TSBV) agar medium and incubated in an atmosphere of 10% CO_2 (Campygen, Oxoid®, Hampshire, UK) during 3–5 days for the identification of *A. actinomycetemcomitans*. Species identification was performed through colony morphology, Gram staining and biochemical and enzymatic assays CAAM test (Slots 1987) to identify *P. gingivalis* and *T. forsythia*. UV was used to differentiate *Prevotella* spp. from *Porphyromonas* spp. (Slots & Reynolds 1982); nitrites and oxidase for *Eikenella corrodens* andcatalase for *A. actinomycetemcomitans*. Positive identifications were subsequently confirmed by the API-ZYM and RAPID ID 32A systems (Biomerieux, Lyon, France).

PCR was performed as described by Ashimoto et al. (1996) and Saiki et al. (1988). The following reference strains were used: *P. gingivalis* (ATCC 33277), *Campylobacter rectus* (ATCC 33238), *T. forsythia* (ATCC 43037), *E. corrodens* (ATCC 23834), *A. actinomycetemcomitans* (ATCC 29522) and *Prevotella intermedia* (ATCC 25611). *P. gingivalis* was identified by the presence of an amplified product in the 404 base-pair (bp) band, *C. rectus* by an amplified product in the 598 bp band, *T. forsythia* in the 641 bp band, *E. corrodens* in the 688 bp band, *P. intermedia* in the 575 bp band. The primers used were those described by Ashimoto et al. (1996), which were selected with the assistance of the Ribosomal Database Project program.

Haemoculture

Each haemoculture was incubated during 2 weeks at 37°C. At 7 and 15 days, subcultures were carried out in agar brucella blood medium enriched with haemin and menadione (BBL Microbiology Systems®) and incubated at 37°C during 7 days in an anaerobic atmosphere (Anaerogen, Oxoid, Hampshire, UK). Bacterial identification was performed in a similar manner as it was described for the subgingival plaque samples. At 3 and 5 days, the haemocultures were assessed for the possible growth of *A. actinomycetemcomitans*. Because no growth was identified, subcultures in agar TSBV were not carried out.

DNA extraction

Immediately after blood sampling, the DNA was extracted from each sample. In order to assure the reliability of this extraction method, we utilized in every patient positive controls (peripheral blood from periodontally healthy subjects contaminated with *P. gingivalis* ATCC 33277) and negative controls (peripheral blood from periodontally healthy subjects).

Total DNA was extracted from the peripheral blood samples using a modified phenol–chloroform purification method, based on the chemical lysis that occurs with extended heating of the samples in the presence of sodium dodecyl sulphate (SDS), hexadecyltrimethyl ammonium bromide (CTAB) and proteinase K. In order to lyse and remove all erythrocytes from the blood sample, 1000 μl of citrated blood was washed with 1000 μl of sterile water and centrifuged at 21,913 *g* for 10 min. at 4°C. The pellets obtained were suspended in 570 μl of Tris EDTA buffer (TE) (1.0 M Tris-HCl pH 8.0; 0.1 M EDTA pH 8.0), and 30 μl of SDS (10% wt/vol) and 3 μl of proteinase K (20 mg/ml) were added. The mixtures were then vigorously mixed by vortexing for 5 min., incubated overnight at 37°C and then subjected to two freeze–thaw cycles (–80°C for 10 min. and heating at 37°C for 10 min.) CTAB and sodium chloride were added to final concentrations of 10% (wt/vol) and 5 M, respectively, and the mixtures were incubated at 65°C for 15 min. to precipitate polysaccharides and residual proteins. Each precipitate was removed by extraction with an equal volume of chloroform/isoamilic alcohol (24:1), followed by phenol–chloroform–isoamilic alcohol (25:24:1). One volume of ice-cold 100% isopropanol was added and the samples were centrifuged at 21,913 *g* for 5 min. The pellet was re-suspended in TE buffer and RNAse (10 mg/ml) was added. After 1 h of incubation at 50°C, DNA was precipitated with sodium acetate (3 M, pH 7.6) and the pellet was purified one more time using the same protocol with chloroform/isoamilic alcohol (24:1), precipitated with ice-cold 95% ethanol, washed twice with ice-cold 70% ethanol, dried and re-suspended in 50 μl of TE buffer. As controls, DNA from cultures of periodontitis-related bacteria was extracted using the same method.

Nested PCR amplification method

To detect the presence of bacterial DNA, we used universal bacterial primers that amplify a conserved region of the 16S ribosomal RNA gene, shared by the vast majority of bacteria. The primer sequences used were EUB-27F: 5′-GAG TTT GAT CCT GGC TCA G-3′ and EUB-1544R: 5′-AGA AAG GAG GTG ATC CAG CC-3′. These primers yielded a 1407 bp PCR products. PCR amplification was carried out in a reaction volume of 100 μl. Each PCR reaction mixture comprised 6 μl of purified DNA and 94 μl of reaction mixture containing 1 × PCR buffer (200 Mm Tris-HCl pH 8.4, 500 mM KCl), 2.0 mM $MgCl_2$, 2,5 U/μl of Taq DNA polymerase (Invitrogen®, Carlsbad, CA. USA), 0.2 mM of each deoxiribonucleotide (Invitrogen®) and 0.5 μM of each primer. DNA amplification was performed with a T-personal Biometra® thermo-cycler (Biometra, Göttingen, Germany) using the following programme: an initial denaturation step at 95°C for 3 min., followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 1 min. and extension at 72°C for 3 min., and then a final extension step at 72°C for 10 min. Four independents replicas were carried out per sample. The purification and concentration of the four replicates were carried out using a purifying PCR DNA Kit (Illustra™GFX™; GE Healthcarelife Science, Uppsala, Sweden) according to the manufacturer's instructions. Positive and negative reaction controls were included for each batch of analysed samples, besides the controls included in the DNA extraction procedure. The positive control used was a standard PCR reaction mixture containing DNA extracted from a blood sample from a healthy subject being contaminated with *P. gingivalis* ATCC 33277, whereas the negative control contained sterile water (Fig. 1). Fifteen microlitres of each PCR products obtained were added to 2 ml of gel loading dye (0.25% bromophenol blue, 50% glycerol, 100 mM EDTA pH 8.0) electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized on a UV transilluminator. A 1 kb DNA ladder (Invitrogen®) was used as a size marker to assist the analysis of the PCR products.

Subsequently, the nested PCR with specific primers for periodontal pathogens was carried out. The primers used for the specific detection of *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythia*, *T. denticola*, *C. rectus*, *E. corrodens* and *P. intermedia* have been reported previously (Ashimoto et al. 1996). Each PCR reaction mixture comprised of 3 μl PCR product and 47 μl of reaction mixture, containing 1 × PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.25 U of Taq DNA polymerase, 0.2 mM of each deoxiribonucleotide (Invitrogen®) and 2μM of each primer. The $MgCl_2$ concentration used was 2.5 mM. DNA amplification was performed with a T-personal Biometra® thermocycler (Biometra). In general, the PCR program consisted on an initial denaturation step of 95°C for 2 min., followed by 35 cycles of denaturation of 95°C for 30 s, primer annealing at 60°C for 1 min. and extension at 72°C for 2 min., and then a final extension step at 72°C for 10 min. The

annealing temperature for *A. actinomycetemcomitans* and *P. intermedia* was 55°C. 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 DNA from pure cultures of the following bacteria: *P. gingivalis* ATTC 33277, *T. forsythia* ATCC 43037, *T. denticola* ATCC 33520, *A. actinomycetemcomitans* DSMZ 8324, *P. intermedia* NCTC 13070, *C. rectus* NCTC 11489 and *E. corrodens* NCTC 10596.

Amplified samples from PCR were electrophoresed through a 1.5% agarose gel in 1 × Tris-acetate EDTA buffer. Fifteen micro-litres of each amplified DNA was added to 2 ml of gel containing 0.25% bromophenol blue, 50% glycerol and 100 mM EDTA at pH 8.0. It was then electrophoresed on a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized on a UV transilluminator. A 100 bp DNA ladder (Invitrogen®) was used as a DNA molecular weight marker. The band position of the PCR products corresponded to the one described by Ashimoto et al. (1996) using the same specific primers.

The positive samples were confirmed by sequencing and comparison with the sequences stored at the GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) using the application tool Basic BLAST de NCBI (National Center for Biotechnology Information).

Statistical analysis

Data were entered in a Microsoft Excel spreadsheet and processed by the software MINITAB 15.0. (Minitab Inc., State College, PA, USA). Descriptive analyses were conducted to assess the distribution of the different clinical variables in the groups studied (frequency distribution for categorical variables and mean and standard deviation for continuous variables). Descriptive analyses also involved the frequency distribution of periodontal pathogens observed in blood samples at different times. χ^2 and Fishers exact tests were used to compare the incidence of bacteraemia between groups (GChP or GAgP) and the bacteria detected in both groups. Statistical significance was set at a p-value < 0.05. The diagnostic validity of nested PCR was assessed by calculating the sensitivity, specificity and likelihood ratios, using the haemoculture data as the gold standard with a 95% confidence. The differences between both techniques were assessed with the χ^2 and Fishers exact tests.

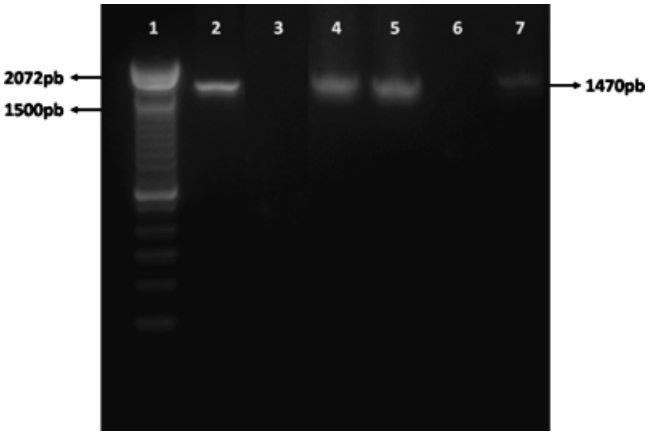

Fig. 1. Agarose gel electrophoresis of a representative polymerase chain reaction result of amplification with 16S ribosomal RNA universal bacterial primers. Lane 1, 1 kb ladder; lane 2, positive control (amplified *Porphyromonas gingivalis* ATCC 33277 DNA); lane 3, negative control; lanes 4–6: amplified peripheral blood samples.

Table 1. Age, gender and clinical parameters in patients treated with scaling and root planing (Lafaurie et al. 2007b)

Parameter	Chronic generalized periodontitis		Aggressive generalized periodontitis	
	total	per cent	total	per cent
Subjects	27/42	64.28%	15/42	35.72%
Gender (%)	F:15	55.5%	F:10	66.7%
	M:12	44.5%	M:5	33.3%
Age (mean ± SD)	42.9 ± 8.3*		33.4 ± 4.9	
PPD (mean ± SD)	3.7 ± 0.9		4.4 ± 0.8*	
CAL (mean ± SD)	3.7 ± 1.4		5.0 ± 1.1*	
Bleeding on probing (% positive)	62.8 ± 26.8		73.9 ± 22.9	
PD-treated sites mm (mean ± SD)	7.4 ± 2.0		8.3 ± 1.8*	
IG-treated sites (% positive)	Grade 1 = 30.5% Grade 2 = 44.0% Grade 3 = 25.5%*		Grade 1 = 31.0% Grade 2 = 57.0% Grade 3 = 12.0%	

*$p < 0.05$ by t-test or χ^2/Fishers exact test.
PD, probing depth; CAL, clinical attachment level; IG, gingival index.

Results

Table 1 depicts the clinical and the socio-demographic data from the patients included in this study. These results were reported previously in the first publication from this investigation (Lafaurie et al. 2007b).

Using universal primers, the complete 16S rRNA gene was amplified from the DNA samples. A representative result depicting the agarose gel electrophoresis of PCR products from blood samples is shown in Fig. 1, with amplicons of approximately 1470 bp. Using specific primers, a representative result showing the detection of *P. gingivalis* is shown in Fig. 2.

From the 168 blood samples analysed in 42 subjects with both techniques, 64/168 (38%) was positive for periodontal pathogens. Twenty-nine (17.2%) of these samples resulted positive by nested PCR for amplification of at least one of the targeted bacteria and 35 (20.8%) were positive by haemoculture. Combining the results from both

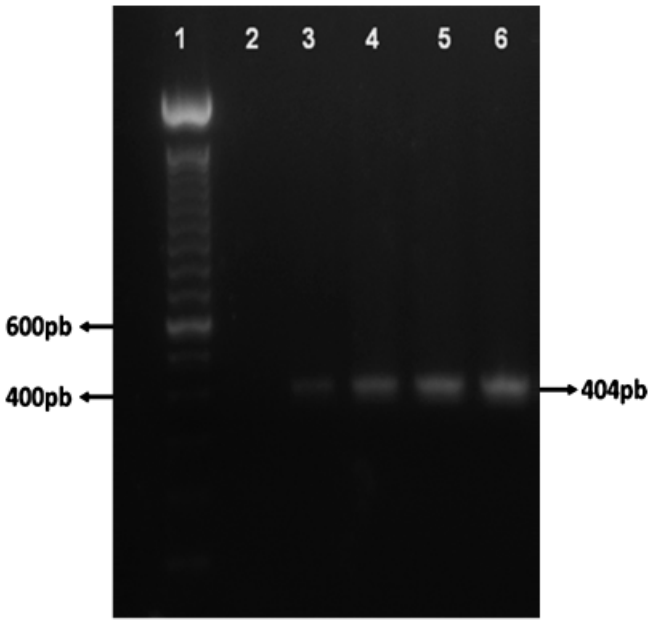

Fig. 2. Agarose gel electrophoresis of representative polymerase chain reaction result of amplification with *Porphyromonas gingivalis* 16S ribosomal RNA specific primers. Lane 1, 100 bp ladder; lane 2, negative control; lane 3, positive control (amplified *P. gingivalis* ATCC 33277 DNA); lanes 4–6, amplified peripheral blood samples.

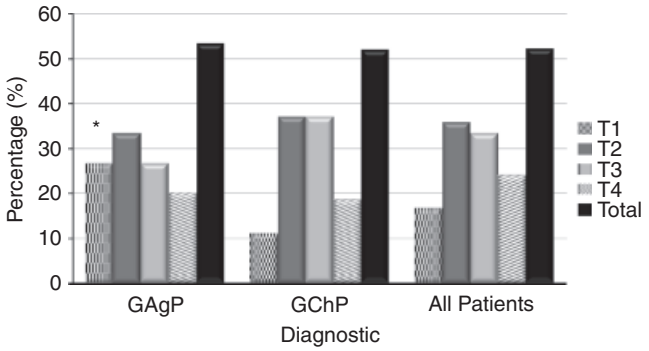

Fig. 3. Prevalence of bacteria in blood samples by nested polymerase chain reaction (nested PCR) and anaerobic culturing [‡]in GChP and GAgP patients at the different sampling times. *Significant differences between T_1 GAgP *versus* T_1 GChP. $p<0.05$ by *t*-test or χ^2/Fishers exact test GAgP: $n=15$, GChP: $n=27$, all patients $n=42$. T_1, pre-treatment; T_2, immediately after treatment; T_3, 15 min. after treatment; T_4, 30 min. after treatment; total, positive at any time after scaling and root planing. Source: Lafaurie et al. 2007(b).

diagnostic techniques (haemoculture and nested PCR) and all collection times after Sc/RP (T_2–T_4), the prevalence of periodontal pathogens was 54.8% (23/42). With nested PCR, this prevalence was 19% (8/42) and with haemoculture 47.6% (20/42). Bacteraemia after Sc/RP was found in 53.3% (8/15) of the GAgP patients and in 51.9% (14/27) of the GChP patients. These differences were not statistically significant ($p>0.05$). In GAgP, however, significantly more microorganisms were detected before therapy (T_1) ($p<0.05$) (Fig. 3).

In 16.6% (7/42) of the patients, pathogens were detected in blood before treatment (T_1), being six of these patients positive by nested PCR and only one by haemoculture. Immediately after treatment (T_2), specific bacterial DNA was detected in 21.4% (9/42) of the subjects, while with haemoculture the percentage of detected bacteria was 38.0% (16/42). In 19% (8/42) of the subjects, bacterial DNA was evident after 15 min. of treatment and 28.6% (12/42) with haemoculture (T_3). After 30 min. post-treatment, in 14.3% (6/42) bacterial DNA was still detected by PCR, while only in 11.9% (5/42) by haemoculture (T_4).

P. gingivalis was detected in subgingival plaque in all patients before Sc/RP and half of the patients in blood after Sc/RP. *P. gingivalis* was the pathogen most frequently detected in blood samples, being identified in (41/168) of the samples, followed by *A. actinomycetemcomitans* (20/168). *P. gingivalis* was detected in higher frequency with nested PCR than with haemoculture, although differences were not significant ($p>0.05$) (Fig. 4, Table 2). Although nested PCR yielded a lower sensitivity to detect patients with *P. gingivalis*, its specificity was, however, higher.

A. actinomycetemcomitans demonstrated the lowest frequency of detection in subgingival plaque samples. Haemoculture was not able to detect this species in bacteraemia samples. With nested PCR, however, *A. actinomycetemcomitans* was the second most detected species. *T. forsythia*, *P. intermedia/nigrenses*, *C. rectus* and *Porphyromonas micra* also demonstrated high detection rates in subgingival plaque samples, but were detected with low frequency in bacteraemia samples. nested PCR slightly improved the detection of *T. forsythia* and *C. rectus*, but not for *P. intermedia*, *P. micra* and *E. corrodens* (Table 2).

Nested PCR demonstrated a higher ability for detecting periodontal pathogens through time. In six serials samples, periodontal pathogens were detected with nested PCR in all times after Sc/RP, while only two samples demonstrated positive bacteria at all times with haemoculture (Table 3).

Discussion

The objective of this study was to evaluate the entry of periodontal pathogens into the bloodstream in subjects with severe periodontal destruction in relation to a therapeutic intervention (Sc/RP) using two microbiological detection techniques (nested PCR and anaerobic blood culturing). The American Heart Association guidelines for prevention of infective endocarditis report that the bacteraemia associated with oral bacteria are often associated with patient's routine activities, such as chewing and toothbrushing and after therapeutic procedures (Wilson et al. 2008). The results obtained in this study support this statement, because a significant percentage of periodontitis patients were positive for periodontal pathogens even before the Sc/RP

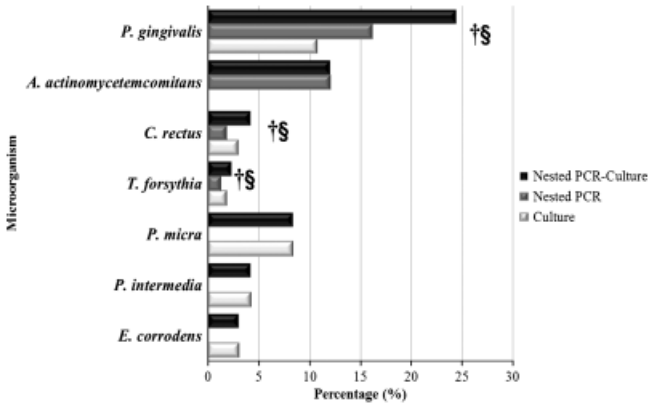

Fig. 4. Prevalence of specific periodontal pathogens in blood samples before and after Sc/RP by nested polymerase chain reaction, anaerobic culturing and combining both techniques ($N = 168$). *Significant differences nested PCR *versus* culture, †significant differences nested PCR culture *versus* culture, §significant differences nested PCR culture *versus* nested PCR, $p < 0.05$ by *t*-test or χ^2/Fishers exact test. ‡Source: Lafaurie et al. 2007(b).

procedure, thus demonstrating that low-level transient bacteraemia may occur in the absence of any therapeutic intervention. Moreover, the presence of periodontal pathogens in peripheral blood samples correlated with disease severity, being more frequent in GAgP patients.

Bacteraemia elicited by haemoculture techniques has been reported as a frequent event in patients with periodontitis, being provoked by different stimuli such as toothbrushing (Lockhart et al. 2008), flossing (Castra et al. 2009) and after therapeutic interventions, mainly mechanical root debridement procedures, such as full-mouth debridement with ultrasonic scalers (Forner et al. 2006) or after standard Sc/RP procedures (Messini et al. 1999). The magnitude of this detection, however, raises concerns, because haemoculture techniques depend on the use of enriched media that may oversize the presence of pathogens in peripheral blood. In the patients of this investigation, we have also reported previously a high prevalence of periodontal pathogens by haemoculture (Lafaurie et al. 2007b). The use of alternative molecular-based detection techniques such as PCR using universal bacterial primers that target the 16S ribosomal RNA genes, has also been reported previously after full-mouth ultrasonic scaling (Kinane et al. 2005), although in very low levels and in a low number of samples. Indeed, when we utilized standard PCR with 16S rRNA genes in these blood samples, we did not obtain any PCR products and only when we amplified with universal primers first and then followed by an nested PCR with specific primers, the identification was positive in a high percentage of the samples tested, which indicates that very low levels of bacteria were present in the analysed samples.

In this study, we have reported with the use of nested PCR with specific primers the presence of specific bacterial DNA, not only after periodontal therapy but also even before any intervention. This specific bacterial DNA was detected in 14% of the patients before the therapeutic intervention and in 19% after Sc/RP. Although haemoculture rendered higher detection rates immediately after the periodontal intervention, this prevalence significantly diminished at subsequent sampling times, while detection by nested PCR was more stable throughout time. This discrepancy may be explained by the fact that haemoculture uses enriched culture media that fosters the growth and proliferation of bacteria, hence resulting in an increased recovery, but not representing the exact amount of microorganisms present at sampling. At subsequent sampling, however, bacterial culture depends on live bacteria and hence, it is likely that pathogens become lysed by the inflammatory and immune response. In contrast, nested PCR will detect bacterial DNA, which is not dependent of cell life or enhanced media.

Detection of DNA from periodontal pathogens, mainly *P. gingivalis*, *A. actinomycetemcomitans* and *C. rectus* has also been reported in vascular lesions, mainly atheromatous plaques (Cairo et al. 2004, Elkaïm et al. 2008) and in placental tissue and amniotic fluid in patients with adverse pregnancy outcomes (León et al. 2007, Bélanger et al. 2008). This detection has elicited the hypothesis on the possible aetiopathogenic role of these pathogens at distant sites. In spite of the fact that only bacterial DNA from periodontal pathogens has been demonstrated in atheroma plaques by PCR or real-time PCR (Fiehn et al. 2005, Kozarov et al. 2006, Gaetti-Jardim et al. 2009) and bacterial cultural techniques were unable to demonstrate the presence of viable bacteria in these lesions (Fiehn et al. 2005), there is evidence of metabolic activity of *P. gingivalis* and *T. denticola* in these lesions, as shown by fluorescence in situ hybridization (FISH) (Cavrini et al. 2005). There is, however, a need of further studies aimed to identify the source of this bacterial DNA present in the atheroma lesions, as well as its possible relationship with the bacteraemia events described in this investigation.

The prevalence of periodontal pathogens in blood has been mostly associated with the presence of *P. gingivalis*. In fact, *P. gingivalis* was the most frequently detected microorganism in this investigation by nested PCR. This finding is similar to results reported using cultural techniques (Forner et al. 2006, Lafaurie et al. 2007b). This pathogen has also been the periodontal pathogen most frequently associated with various cardiovascular diseases (Gaetti-Jardim et al. 2009). It has been proven experimentally that *P. gingivalis* can adhere and invade epithelial cells in vitro (Amano 2003, Saito et al. 2008), may induce atherosclerotic lesions under in vivo conditions (Brodala et al. 2005, Kubota et al. 2008) and can induce vascular changes through the inflammatory pathways involved in atherogenesis (Champagne et al. 2009).

P. micra and *A. actinomycetemcomitans* showed a low recovery in subgingival plaque samples. This finding agrees with previous investigations assessing the composition of subgingival plaque in patients with chronic periodontitis in Colombia (Botero et al. 2007, Lafaurie et al. 2007a). In fact, in a comparative study between patients from Chile and Spain, the frequency of *P. micra* from Colombia was significantly lower (Herrera et al. 2008). *P. micra* is, however, an oral microorganism associated with cervicofacial abscesses (Araki et al. 2004) and extraoral infections (Civen et al. 1995).

Table 2. Frequency of periodontal pathogens in subgingival plaque and peripheral blood

Microorganims	Subgingival plaque	Periodontopatic microorganisms in peripheral blood					likelihood ratio	
		nested PCR and culture†	culture†	nested PCR	PCR sensitivity gold standard culture† (%)	PCR specificity gold standard culture† (%)	positive	negative
P gingivalis	42/42 (100%)	21/42 (50%)	12/42 (28.6%)	13/42 (31%)	38.1 (6.3–18.5)	95 (84.7–90.8)	5.17 (0.78–2.01)	1.08 (0.74–0.90)
Eikenella corrodens	42/42 (100%)	4/42 (9.5%)	4/42 (9.5%)	0/42 (0%)	NC	NC	NC	NC
Tannerella forsythia	41/42 (97.6%)	5/42 (11.9%)	3/42* (7.1%)	2/42* (4.8%)	84.2 (0–0)	99.6 (94.8–98.2)	NC	1.4 (1–1.04)
Prevotella intermedia	40/42 (95.2%)	3/42 (7.1%)	3/42 (7.1%)	0/42 (0%)	NC	NC	NC	NC
Campylobacter rectus	38/42 (90.5%)	7/42 (16.16.6)	5/42* (11.9)	2/42* (4.8)	70.8 (0–0)	99.0 (93.0–97.0)	NC	1.06 (1–1.03)
Porphyromonas micra	29/42 (28.6%)	7/42 (16.7%)	7/42 (16.7%)	0/42 (0%)	NC	NC	NC	NC
Aggregatibacter actinomycetemcomitans	11/42 (26.2%)	9/42 (21.4%)	0/42 (0%)	9/42 (21.4%)	NC	NC	NC	NC

*Significant differences in the comparison between nested PCR culture. $p<0.05$ by *t*-test or χ^2/Fishers exact test.
†Data extracted from Lafaurie et al. (2007b).
NC, no calculate; nested PCR, nested polymerase chain reaction.

The mechanisms by which these oral microorganisms cause distance infections may be due to their ability to survive in blood during bacteraemia. *A. actinomycetemcomitans* was the second most frequently detected periodontal pathogen in peripheral blood before and after Sc/RP treatment with nested PCR. *A. actinomycetemcomitans* has been associated with infective endocarditis (Paturel et al. 2004), being part of the HACEK Group (fastidious organisms associated with infective endocarditis) (Berbari et al 1997). Infection with *A. actinomycetemcomitans* has also been associated with cardiovascular events. High antibodies titres against *A. actinomycetemcomitans* were significantly associated with acute coronary syndrome (Pussinen et al. 2005, Sakurai et al. 2007). Anaerobic culturing, however, has not yielded a positive identification of this pathogen in most bacteraemia studies (Messini et al 1999, Kinane et al 2005, Forner et al 2006, Lafaurie et al 2007b), being detected in just one patient by Castra et al. (2009) after flossing and also in one patient by Lockhart et al. (2008) after toothbrushing. This fact may be due to the difficulties inherent to the cultivation of *A. actinomycetemcomitans* requiring high concentrations of CO_2 and the use of selective media, such as the TSVB (Slots 1982) or the Dentaid-1 medium (Alsina et al. 2001). It is likely that the anaerobic haemoculture methods currently used do not allow the growth of this bacteria or that high numbers are required in order to yield a positive identification. Further research should be carried out to evaluate the detection of *A. actinomycetemcomitans* in blood samples with the use of selective media and with a microaerophilic environment.

T. forsythia and *C. rectus* were also detected in peripheral blood by nested PCR, although in less frequency. These organisms were detected more frequently by blood culture. *E. corrodens* and *P. intermedia* were not detected by molecular techniques, although they could be detected by anaerobic culturing. The amounts of these bacteria in blood are probably in such a low number that even PCR is unable to detect them.

In spite of a high prevalence of *P. intermedia* in subgingival plaque, the presence of this pathogen in peripheral blood with both diagnostic techniques was very low. This finding has also been reported by other authors in bacteraemia studies (Kinane et al. 2005, Bahrani-Mougeot et al. 2008, Lockhart et al. 2008, Castra et al. 2009). This discrepancy may be due to the high sensitivity to oxygen presented by bacteria from the genus *Prevotella*, which will reduce their survival in peripheral blood or may cause mutations in their DNA.

The results from this investigation show that nested PCR may be an adequate diagnostic method to supplement haemoculture in bacteraemia studies, although these findings only provide indirect evidence of the presence of the pathogens, because molecular-based diagnostic techniques are only able to detect DNA rather than live bacteria (Takeuchi et al. 1999, Takumi et al. 2008). These results are in agreement with a recent systematic review assessing the utility of molecular-based diagnosis for the study of pathogens associated with sepsis and septic shock. This study concluded that PCR methods, including nested PCR and real-time PCR do not have the necessary sensitivity and specificity for being utilized in the clinic, and therefore, cannot yet replace culture. These molecular techniques based on bacterial DNA detection in blood may be hampered by the presence of human DNA and DNA inhibitors in blood, thus rendering frequent hybridization reactions as well as false negative results (Avni et al. 2010).

In conclusion, the use of nested PCR demonstrated the presence of DNA from periodontal pathogens in blood samples in severe periodontitis patients before, during and after periodontal therapy. The number of pathogens in blood significantly increased with treatment, although they could be still detected 30 min. after the intervention. The use of these molecular-based techniques for the detection of periodontal pathogens in blood samples may improve the accuracy from the results obtained by

?*Table 3*. Periodontopatic microorganisms isolated from GChP and GAgP patients in blood cultures and nested PCR at different times

Patient	Diagnostic	T_1		T_2		T_3		T_4	
		culture*	nested PCR	culture*	nested PCR	culture*	nested PCR	culture*	nested PCR
1	GAgP			*Porphyromonas gingivalis*	*P. gingivalis* *Tannerella forsythia* *Aggregatibacter actinomycetemcomitans*		*P. gingivalis* *A. actinomycetemcomitans*		*P. gingivalis* *A. actinomycetemcomitans*
2	GChP			*Eikenella corrodens*		*E. corrodens*		*E. corrodens*	
8	GAgP							*P. gingivalis*	
9	GChP			*P. gingivalis* *Campylobacter* spp.		*P. gingivalis*			
12	GAgP					*P. gingivalis*	*P. gingivalis* *T. forsythia* *C. rectus*		
13	GChP			*P. gingivalis*	*P. gingivalis* *C. rectus* *A. actinomycetemcomitans*		*P. gingivalis* *C. rectus* *A. actinomycetemcomitans*		
14	GChP				*P. gingivalis* *A. actinomycetemcomitans*				
15	GAgP		*P. gingivalis* *A. actinomycetemcomitans*	*Porphyromonas micra*	*P. gingivalis* *A. actinomycetemcomitans*	*P. micra*	*P. gingivalis* *A. actinomycetemcomitans*	*P. micra*	*P. gingivalis* *A. actinomycetemcomitans*
16	GChP		*P. gingivalis* *A. actinomycetemcomitans*		*P. gingivalis* *A. actinomycetemcomitans*		*P. gingivalis* *A. actinomycetemcomitans*		
17	GChP				*P. gingivalis* *A. actinomycetemcomitans*		*P. gingivalis* *A. actinomycetemcomitans*		*P. gingivalis*
18	GChP					P. micra			
19	GChP			*P. micra*					
20	GChP			*P. gingivalis*		*P. gingivalis*			
27	GChP					*P. gingivalis* *T. forsythia*		*P. gingivalis*	
28	GAgP		*P. gingivalis*	*P. gingivalis*	*P. gingivalis*	*P. gingivalis* *P. micra*	*P. gingivalis* *A. actinomycetemcomitans*		*P. gingivalis*
30	GChP			*E. corrodens*		*P. gingivalis*			
31	GAgP	*Campylobacter* spp. *P. intermedia*							
32	GAgP			*T. forsythia* *P. intermedia*					
33	GAgP			*P. gingivalis*					
34	GAgP					*T. forsythia*			
35	GAgP		*P. gingivalis* *A. actinomycetemcomitans*		*A. actinomycetemcomitans*				*P. gingivalis*
36	GAgP			*P. gingivalis* *P. intermedia*					
37	GChP		*P. gingivalis* *A. actinomycetemcomitans*	*Campylobacter* spp. *E. corrodens*	*P. gingivalis* *A. actinomycetemcomitans*				
38	GAgP			*Campylobacter* spp.			*P. gingivalis*	*P. micra*	
39	GChP								*P. gingivalis*
40	GChP			*P. micra* *Campylobacter* spp.		*P. gingivalis* *E. corrodens*			
42	GChP		*P. gingivalis*						

GChP, generalized severe chronic periodontitis; GAgP, generalized aggressive periodontitis.
*Source: Lafaurie et al. (2007b).

haemoculture and in this way it could help us to understand their possible pathogenic role in the proven association between periodontitis and cardiovascular diseases.

References

Ali, R. W., Velcescu, C. & Jivanescu, M. C. (1996) Prevalence of 6 putative periodontal pathogens in subgingival plaque samples from Romanian adult periodontitis patients. *Journal of Clinical Periodontology* **23**, 133–139.

Alsina, M., Olle, E. & Frias, J. (2001) Improved, low-cost selective culture medium for *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Microbiology* **39**, 509–513.

Amano, A. (2003) Molecular interaction of *Porphyromonas gingivalis* with host cells: implication for the microbial pathogenesis of periodontal disease. *Journal of Periodontology* **74**, 90–6.

Araki, H., Kuriyama, T., Nakagawa, K. & Karasawa, T. (2004) The microbial synergy of *Peptostreptococcus micros* and *Prevotella intermedia* in a murine abscess model. *Oral Micriobiology and Immunology* **19**, 177–181.

Armitage, G. C. (1999) Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology* **4**, 1–6.

Ashimoto, A., Chen, C., Bakker, I. & Slots, J. (1996) Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lessions. *Oral Microbiology and Immunology* **11**, 266–273.

Avni, T., Mansur, N., Leibovici, L. & Paul, M. (2010) PCR using blood for diagnosis of invasive pneumococcal disease: systematic review and meta-analysis. *Journal of Clinical Microbiology* **48**, 489–496.

Bahrani-Mougeot, F., Paster, B., Coleman, S., Ashar, J., Barbuto, S. & Lockhart, P. (2008) Diverse and novel oral bacterial species in blood following dental procedures. *Journal of Clinical Microbiology* **46**, 2129–2132.

Bélanger, M., Reyes, L., von Deneen, K., Reinhard, M. K., Progulske-Fox, A. & Brown, M. B. (2008) Colonization of maternal and fetal tissues by *Porphyromonas gingivalis* is strain-dependent in a rodent animal model. *American Journal of Obstetrics Gynecology* **199**, e1–e7.

Berbari, E. F., Cockerill, F. R. III & Steckelberg, J. M. (1997) Infective endocarditis due to unusual or fastidious microorganisms. *Mayo Clinic Proceedings* **72**, 532–42.

Botero, J. E., Contreras, A., Lafaurie, G., Jaramillo, A., Betancourt, M. & Arce, R. M. (2007) Occurrence of periodontopathic and superinfecting bacteria in chronic and aggressive periodontitis subjects in a Colombian population. *Journal of Periodontology* **78**, 696–704.

Brodala, N., Merricks, E. P., Bellinger, D. A., Damrongsri, D., Offenbacher, S., Beck, J., Madianos, P., Sotres, D., Chang, Y. L., Koch, G. & Nichols, T. C. (2005) *Porphyromonas gingivalis* bacteremia induces coronary and aortic atherosclerosis in normocholesterolemic and hypercholesterolemic pigs. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25**, 1446–51.

Cairo, F., Gaeta, C., Dorigo, W., Oggioni, M. R., Pratesi, C., Pini Prato, G. P. & Pozzi, G. (2004) Periodontal pathogens in atheromatous plaques. A controlled clinical and laboratory trial. *Journal of Periodontal Research* **39**, 442–6.

Castra, K., Daly, C. G., Mitchell, D., Curtis, B., Stewart, D. & Heitz-Mayfield, L. J. (2009) Bacteraemia due to dental flossing. *Journal of Clinical Periodontology* **36**, 323–32.

Cavrini, F., Sambri, V., Moter, A., Servidio, D., Marangoni, A., Montebugnoli, L., Foschi, F., Prati, C., Di Bartolomeo, R. & Cevenini, R. (2005) Molecular detection of *Treponema denticola* and *Porphyromonas gingivalis* in carotid and aorti-catheromatous plaques by FISH: report of two cases. *Journal of Medical Microbiology* **54**, 93–96.

Champagne, C., Yoshinari, N., Oetjen, J. A., Riché, E. L., Beck, J. D. & Offenbacher, S. (2009) Gender differences in systemic inflammation and atheroma formation following *Porphyromonas gingivalis* infection in heterozygous apolipoprotein E-deficient mice. *Journal of Periodontal Research* **44**, 569–77.

Civen, R., Jousimies-Somer, H., Marina, M., Borenstein, L., Shah, H. & Finegold, S. M. (1995) A retrospective review of cases of anaerobic empyema and update of bacteriology. *Clinical Infectious Diseases* **20** (Suppl. 2), S224–S229.

Dzink, J. L., Socransky, S. S. & Haffajee, A. D. (1988) The predominant cultivable microbiota of active and inactive lesions of destructive periodontal disease. *Journal of Clinical Periodontology* **15**, 161–168.

Elkaïm, R., Dahan, M., Kocgozlu, L., Werner, S., Kanter, D., Kretz, J. G. & Tenenbaum, H. (2008) Prevalence of periodontal pathogens in subgingival lesions, atherosclerotic plaques and healthy blood vessels: a preliminary study. *Journal of Periodontal Research* **43**, 224–231.

Fiehn, N. E., Larsen, T., Christiansen, N., Holmstrup, P. & Schroeder, T. V. (2005) Identification of periodontal pathogens in atherosclerotic vessels. *Journal of Periodontology* **76**, 731–736.

Forner, L., Larsen, T., Kilian, M. & Holmstrup, P. (2006) Incidence of bacteremia after chewing, tooth brushing and scaling in individuals with periodontal inflammation. *Journal of Clinical Periodontology* **33**, 401–407.

Gaetti-Jardim, E. Jr, Marcelino, S. L., Feitosa, A. C., Romito, G. A. & Avila-Campos, M. J. (2009) Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries. *Journal of Medical Microbiology* **58**, 1568–1575.

Geerts, S. O., Nys, M., De, M. P., Charpentier, J., Albert, A., Legrand, V. & Rompen, E. H. (2002) Systemic release of endotoxins induced by gentle mastication: association with periodontitis severity. *Journal of Periodontology* **73**, 73–78.

Haffajee, A. D., Socransky, S. S., Patel, M. R. & Song, X. (2008) Microbial complexes in supragingival plaque. *Oral Microbiology and Immunology* **23**, 196–205.

Herrera, D., Contreras, A., Gamonal, J., Oteo, A., Jaramillo, A., Silva, N., Sanz, M., Botero, J. E. & León, R. (2008) Subgingival microbial profiles in chronic periodontitis patients from Chile, Colombia and Spain. *Journal of Clinical Periodontology* **35**, 106–113.

Ide, M., Jagdev, D., Coward, P. Y., Crook, M., Barclay, G. R. & Wilson, R. F. (2004) The short-term effects of treatment of chronic periodontitis on circulating levels of endotoxin, C-reactive protein, tumor necrosis factor-alpha, and interleukin-6. *Journal of Periodontology* **75**, 420–428.

Kinane, D. F., Riggio, M. P., Walker, K. F., MacKenzie, D. & Shearer, B. (2005) Bacteremia following periodontal procedures. *Journal of Clinical Periodontology* **32**, 708–713.

Kozarov, E., Sweier, D., Shelburne, C., Progulske-Fox, A. & Lopatin, D. (2006) Detection of bacterial DNA in atheromatousplaques by quantitative PCR. *Microbes and Infection* **8**, 687–693.

Kubota, T., Inoue, Y., Iwai, T., Kurihara, N., Huang, Y. & Umeda, M. (2008) Arterial thrombosis after intravenous infusion of oral bacterium in a rat model. *Annals of Vascular Surgery* **22**, 412–416.

Lafaurie, G. I., Contreras, A., Barón, A., Botero, J., Mayorga-Fayad, I., Jaramillo, A., Giraldo, A., González, F., Mantilla, S., Botero, A., Archila, L. H., Díaz, A., Chacón, T., Castillo, D. M., Betancourt, M., Del Rosario Aya, M. & Arce, R. (2007a) Demographic, clinical, and microbial aspects of chronic and aggressive periodontitis in Colombia: a multicenter study. *Journal of Periodontology* **78**, 629–639.

Lafaurie, G. I., Mayorga-Fayad, I., Torres, M. F., Castillo, D. M., Aya, M. R., Barón, A. & Hurtado, P. A. (2007b) Periodontopathic microorganisms in peripheric blood after scaling and root planing. *Journal of Clinical Periodontology* **34**, 873–879.

León, R., Silva, N., Ovalle, A., Chaparro, A., Ahumada, A., Gajardo, M., Martinez, M. & Gamonal, J. (2007) Detection of *Porphyromonas gingivalis* in the amniotic fluid in pregnant women with a diagnosis of threatened premature labor. *Journal of Periodontology* **78**, 1249–1255.

Lockhart, P. B., Brennan, M. T., Sasser, H. C., Fox, P. C., Paster, B. J. & Bahrani-Mougeot, F. K. (2008) Bacteremia associated with toothbrushing and dental extraction. *Circulation* **117**, 3118–3125.

Messini, M., Skourti, I., Markopulos, E., Koutsia, C., Kyriakopoulou, E., Kostaki, S., Lambraki, D. & Georgopoulos, A. (1999) Bacteremia after dental treatment in mentally handicapped people. *Journal of Clinical Periodontology* **26**, 469–473.

Mustapha, I. Z., Debrey, S., Oladubu, M. & Ugarte, R. (2007) Markers of systemic bacterial exposure in periodontal disease and cardiovascular disease risk: a systematic review and meta-analysis. *Journal of Periodontology* **78**, 2289–2302.

Nakano, K., Nemoto, H., Nomura, R., Inaba, H., Yoshioka, H., Taniguchi, K., Amano, A. & Ooshima, T. (2009) Detection of oral bacteria in cardiovascular specimens. *Oral Microbiology and Immunology* **24**, 64–68.

Paturel, L., Casalta, J. P., Habib, G., Nezri, M. & Raoult, D. (2004) *Actinobacillus actinomycetemcomitans* endocarditis. *Clinical Microbiology and Infection* **10**, 98–118.

Pussinen, P. J., Nyyssönen, K., Alfthan, G., Salonen, R., Laukkanen, J. A. & Salonen, J. T. (2005) Serum antibody levels to *Actinobacillus actinomycetemcomitans* predict the risk for coronary heart disease. *Arteriosclerosis, Thrombosis and Vascular Biology* **25**, 833–838.

Saiki, R. K., Gelfand, D. H., Stoffel, . S., Scharf, . S. J., Higuchi, . R., Horn, . G. T., Mullis, K. B. & Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **29**, 487–491.

Saito, A., Inagaki, S., Kimizuka, R., Okuda, K., Hosaka, Y., Nakagawa, T. & Ishihara, K. (2008) *Fusobacterium nucleatum* enhances invasion of human gingival epithelial and aortic endothelial cells by *Porphyromonas gingivalis*. *FEMS Immunology and Medical Microbiology* **54**, 349–355.

Sakurai, K., Wang, D., Suzuki, J., Umeda, M., Nagasawa, T., Izumi, Y., Ishikawa, I. & Isobe, M. (2007) High incidence of *Actinobacillus actinomycetemcomitans* infection in acute coronary syndrome. *International Heart Journal* **48**, 663–675.

Slots, J. (1987) Detection of colonies of bacteroides gingivalis by a rapid fluorescence assay for trypsin-like activity. *Oral Microbiology and Immunology* **2**, 139–141.

Slots, J. (1982) Selective Medium for Isolation of *Actinobacillus actinomycetemcomitans*. *Journal of Clinical Microbiology* **15**, 606–609.

Slots, J. & Reynolds, H. S. (1982) Long-wave UV light fluorescence for identification of black pigmented bacteroides. *Oral Microbiology and Immunology* **16**, 1148–1151.

Takeuchi, T., Nakaya, Y., Kato, N., Watanabe, K. & Morimoto, K. (1999) Induction of oxidative DNA damage in anaerobes. *FEBS Letters* **450**, 178–180.

Takumi, S., Komatsu, M., Aoyama, K., Watanabe, K. & Takeuchi, T. (2008) Oxygen induces mutation in

a strict anaerobe, *Prevotella melaninogenica*. *Free Radical Biology & Medicine* **44**, 1857–1862.

Wilson, W., Taubert, K. A., Gewitz, M., Lockhart, P. B., Baddour, L. M., Levison, M., Bolger, A., Cabell, C. H., Takahashi, M., Baltimore, R. S., Newburger, J. W., Strom, B. L., Tani, L. Y., Gerber, M., Bonow, R. O., Pallasch, T., Shulman, S. T., Rowley, A. H., Burns, J. C., Ferrieri, P., Gardner, T., Goff, D., Durack, D. T. & American Heart Association. (2008) Prevention of infective endocarditis: guidelines from the American Heart Association: a guideline from the American Heart Association Rheumatic Fever, Endocarditis and Kawasaki Disease Committee, Council on Cardiovascular Disease in the Young, and the Council on Clinical Cardiology, Council on Cardiovascular Surgery and Anesthesia, and the Quality of Care and Outcomes Research Interdisciplinary Working Group. *Journal of American Dental Association* **139** (Suppl.), 3S–24S.

Address:
Gloria I. Lafaurie
Faculty of Dentistry
El Bosque University
Transversal 9A Bis No 133-55
Bogota
Colombia
E-mail: investigaodonto@unbosque.edu.co

Clinical Relevance

Scientific rationale for the study: The entry of periodontal pathogens to the bloodstream through the gingival–biofilm interface has been proposed as one of the key pathogenic mechanisms associated in the possible link between periodontal infections and systemic diseases. For detecting and identifying these pathogens, haemoculture has been the most widely used methodology, but its reliability is hampered by the need to use enriched culture media. Molecular-based diagnostics such as nested PCR could improve the accuracy in the detection of these bacteria in blood samples, and therefore, could provide us a more reliable diagnosis on the presence or absence of bacteraemia in patients with periodontitis and its relationship with either daily event, such as toothbrushing or to therapeutic interventions.

Principal findings: This study has shown that the use of specific nested PCR for subgingival periodontal pathogens is able to detect these pathogens in blood samples and hence complements the information obtained with haemoculture. *P. gingivalis* and *A. actinomycetemcomitans* were the most frequently identified bacteria. Although periodontal pathogens were identified in peripheral blood more frequently after the periodontal intervention, they were also detected, albeit in less numbers, in samples drawn before this intervention.

Practical implications: Bacteraemia by periodontal pathogens in periodontitits patients may pose these patients in risks of systemic complications if these bacteria are able to colonize and cause damage at distance locations or elicit a significant chronic systemic inflammatory response. The use of molecular-based microbial detection methods in peripheral blood may help to understand the frequency and clinical relevance of this systemic exposure.