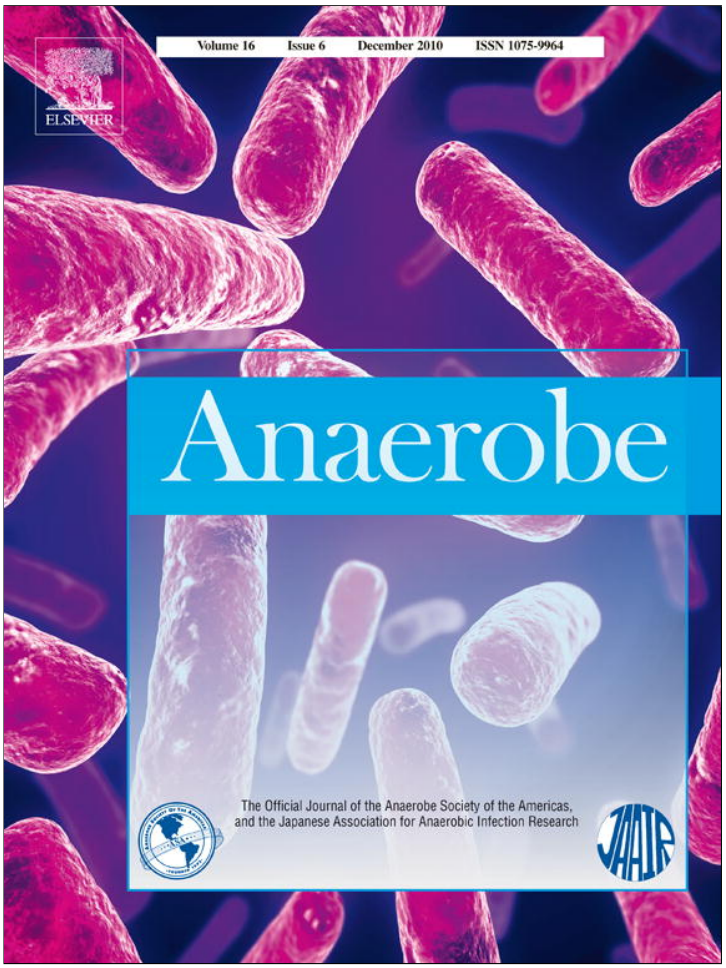

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Note

Presence of periodontopathic bacteria in coronary arteries from patients with chronic periodontitis

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ABSTRACT

In this study the presence of periodontopathic pathogens in atheromatous plaques removed from coronary arteries of patients with chronic periodontitis and periodontally healthy subjects by PCR was detected. Our results indicate a significant association between the presence of *Porphyromonas gingivalis* and atheromas, and the periodontal bacteria in oral biofilm may find a way to reach arteries.

1. Introduction

Periodontitis is a chronic polymicrobial infection determined by the interaction between microorganisms and immune, environmental, behavioral and/or hereditary factors, causing an inflammatory response of the periodontal tissues [1,2]. This disease affects individuals of different ages and is characterized by a chronic tissue-destructive inflammation and dental attachment loss [3]. The pathogenesis of periodontitis is a result of the accumulation of bacterial species in subgingival biofilm, particularly by Gram-negative anaerobic and microaerophilic bacteria, such as *Porphyromonas gingivalis* (*Pg*), *Prevotella intermedia* (*Pi*), *Prevotella nigrescens* (*Pn*), *Tannerella forsythia* (*Tf*), *Treponema denticola* (*Td*), *Fusobacterium nucleatum* (*Fn*), *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Campylobacter rectus* (*Cr*) [1]. Experimental data support the association between the virulence of periodontal microorganisms, such as their ability to induce platelet aggregation and foam-cell formation, and their involvement in the development of atheroma [4].

2. Materials and methods

2.1. Patients

In this study, 30 patients were examined; 28 patients displaying chronic periodontitis and two periodontally healthy individuals (mean age 61 ± 11.5 years) were selected from March 2005 to March 2007 at the Evangelic Hospital (Londrina, PR, Brazil). All patients presented diffuse atherosclerotic disease, multiple stenoses with distal and diffuse involvement with reversible ischemia, documented by scintigraphy and analysis of echocardiography in conditions considered inoperable by conventional methods, or advanced atherosclerotic disease with viable myocardium.

Patients were included if they had at least 6 teeth and clinical attachment loss in 30% of the existing teeth [5]. None of the patients received any antibiotic therapy in the previous 3 months. Patients with systemic diseases, diabetes or severe acute apical periodontitis were excluded. All the patients gave their written informed consent to participate in this study which was approved by the Ethics Committee of the Institute of Biomedical Science, USP (Protocol. 270/ CEP).

All patients underwent a clinical interview in order to obtain information about their identification, age, and disease, medical and familial histories. In addition, 7 days prior endarterectomy of

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Table 1
Bacterial target, primer sequences, annealing temperature and amplicon predicted used in the PCR detection.

Bacterial target	Primer sequences (5′ → 3′)	Annealing temperature (°C)	Amplicon (bp)	References
A. actinomycetemcomitans	AAA CCC ATC TCT GAG TTC TTC TTC ATG CCA ACT TGA AGT TAA AT	56	557	[7]
C. rectus	TTT CGG AGC CAT ACG TCC TAA G TTT CTG CAA GCA GAC ACT CTT	50	598	[7]
E. faecalis	TAG TGA CAA ACC ATT CAT GAA TG AAC TTC GTC ACC AAC GCG AAC	55	112	[9]
F. nucleatum	ATT GTG GCT AAA AAT TAT AGT T ACC CTC ACT TTG AGG ATT ATA G	40	500	[8]
P. endodontalis	GCT GCA GCT CAA CTG TAG TC CCG CTT CAT GTC ACC ATC TC	60	672	[7]
P. gingivalis	AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	60	404	[7]
P. intermedia	TTT GTT GGG GAG TAA AGC GGG TCAA CAT CTC TGT ATC CTG CGT	50	575	[7]
P. nigrescens	ATG AAA CAA AGG TTT TCC GGT AAG CCC ACG TCT CTG TGG GCT GCG A	55	804	[7]
T. forsythia	GCC TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	60	641	[7]
T. denticola	TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	55	316	[7]
Universal Primers 16S rRNA	AGA GTT TGA TCC TGG CTC AG CAA TAC TCG TAT CGC CCG TTA TTC	60	1500	[6]

coronary arteries, patients were submitted to a complete periodontal examination at the Evangelic Hospital, collecting data on plaque index (full-mouth plaque score), bleeding on probing (full-mouth bleeding score), probing depth (PD), gingival recession (GR) and clinical attachment level (CAL) at six sites (mesial, mid, and distal sites of oral and facial surfaces) per tooth, excluding third molars, using a periodontal probe PCP UNC-15 (Hu-Friedy, Chicago, IL). In order to evaluate the presence of periapical infections or bone lesions, radiographic examination of the maxilla and mandible was carried out, and dental pulp viability was assessed by using an electric pulp test.

2.2. DNA extraction

Atheromatous plaques from coronary arteries were collected during endarterectomy and placed in vials containing 5 ml RNAlater (Ambion, Applied Biosystem, Foster, CT, USA) and stored at −20 °C until DNA preparation. Approximately 100 mg tissue was subjected to DNA extraction using a Charge Switch gDNA Mini Tissue Kit (Invitrogen™ Brasil Ltd., São Paulo, Brazil). Subgingival biofilm was collected from all patients by using sterile paper points, which were introduced into the deepest periodontal pockets with clinical evidences of bleeding on probing. After 30 s, the periodontal samples were transferred to 300 μL ultrapure water and then, genomic DNA was extracted using the methodology described above.

2.3. Bacterial detection by PCR

The primers utilized were designed in accordance to Amano et al. [6], Ashimoto et al. [7], Avila-Campos et al. [8] and Ke et al. [9] (Table 1) and were synthesized by Invitrogen™ Brasil (São Paulo, SP, Brazil). PCR amplification was carried out in a 25 μL reaction mix containing 1× PCR buffer, 50 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer pair, 0.5 U *Taq* Platinum DNA polymerase (Invitrogen) and 10 ng of DNA. Amplification was carried out in a thermal cycle (GeneAmp PCR System 9700), programmed at 94 °C (5 min) followed by 30 cycles at 94 °C (30 s). Annealing temperature for each primer pair (Table 1) for 30 s, and then 72 °C for 30 s, then 72 °C for 5 min to allow the completion of DNA extension.

A negative control reaction without DNA was included in each PCR run. The strains *P. gingivalis* ATCC 33277, *P. endodontalis* ATCC 35406, *P. intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, *T. forsythia* ATCC 43037, *T. denticola* ATCC 33520, *F. nucleatum* ATCC 25611, *A. actinomycetemcomitans* ATCC 29523, *C. rectus* ATCC 33238 and *E. faecalis* ATCC 33563 were used as control for genomic DNA. The PCR products were analyzed by electrophoresis in 1% agarose gel, stained with ethidium bromide (0.5 μg/ml), and photographed under ultraviolet light.

In order to evaluate the specificity of the DNA amplifications to each positive clinical sample, the PCR products were excised and purified, and the 16S rRNA gene fragment was amplified in accordance Song et al. [10], using a QIAquick gel extraction kit (Qiagen) for sequencing by using a Biotech Diagnostics big-Dye sequencing kit on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, Calif). The sequencing data was analyzed by comparison of the consensus sequences with GenBank and BLAST [11,12].

3. Results

All atheroma samples harbored bacterial DNA detected by universal primers, and all reference strains DNA produced the expected amplicons by using the species-specific primers. DNA sequencing analysis of each bacterium from positive samples showed a similarity of 95–98%. The detection rates for the bacterial species in atheromatous plaques and subgingival biofilm, as well as the presence of bacterial associations in the atheromas are reported in Tables 2 and 3, respectively.

In the statistical analysis, median values and standard deviations were calculated for each bacterium and the tests were performed using the software Statistical Package for the Social Sciences (SPSS Inc v.13, Chicago, IL, USA). Differences among clinical and microbiological parameters were evaluated using the Mann–Whitney or Fisher's exact tests. Difference of $p < 0.05$ was considered statistically significant. The mean number of teeth was 14.9 ± 6.72 for patients with chronic periodontitis and the mean values for the clinical parameters were as follows: PD = 5.8 ± 0.9, GR = 2.2 ± 0.34 and CAL = 6.2 ± 1.2 mm. Healthy subjects showed a mean number of teeth of 18.5 ± 4.3 and the mean values for

Table 2
Presence of periodontopathic bacteria in coronary atheromatous plaques isolated from patients with chronic periodontitis.

Microorganism	Patients with periodontitis ($N = 28$)		Periodontally healthy patients ($N = 2$)	
	AP[a], N(%)	PS[b], N(%)	AP, N(%)	PS, N(%)
P. gingivalis	14 (50.0)	21 (75.0)	1 (50.0)	1 (50.0)
P. intermedia	5 (17.9)	17 (60.7)	0 (0.0)	1 (50.0)
E. faecalis	4 (14.3)	8 (28.6)	1 (50.0)	1 (50.0)
P. nigrescens	4 (14.3)	7 (25.0)	1 (50.0)	1 (50.0)
A. actinomycetemcomitans	2 (7.1)	7 (25.0)	0 (0.0)	0 (0.0)
C. rectus	2 (7.1)	15 (53.6)	0 (0.0)	1 (50.0)
T. forsythia	2 (7.1)	16 (57.1)	0 (0.0)	0 (0.0)
P. endodontalis	1 (3.6)	11 (39.3)	0 (0.0)	0 (0.0)
T. denticola	1 (3.6)	6 (21.4)	0 (0.0)	0 (0.0)
F. nucleatum	0 (0.0)	23 (82.1)	0 (0.0)	1 (50.0)
Total of positive samples for any targeted periodontal bacteria	19 (67.9)	28 (100.0)	1 (50.0)	2 (100.0)
Total microbial DNA[c]	28 (100.0)	28 (100.0)	2 (100.0)	2 (100.0)

[a] Atheromathous plaques.
[b] Periodontal samples.
[c] Data obtained by using universal 16S rRNA primers.

clinical parameters were as follows: PD = 1.5 ± 0.9, GR = 0.6 ± 0.22 and CAL = 1.3 ± 0.22 mm.

Selected periodontal bacteria were detected in 67.9% of the atherosclerotic samples from patients with periodontitis. However, due to the reduced number of atheromatous samples from periodontally healthy subjects, it was not possible to compare the influence of the periodontal status on the prevalence of selected microorganisms in atheromas, but it was noticed a clear association between the presence of *P. gingivalis* in the periodontal pockets and in atheromatous tissues (Fisher's exact test, $p = 0.012$), but no association with the other bacteria was observed.

The occurrence of the periodontal microorganisms in subgingival biofilm of the evaluated population shows a statistically significant association between *T. forsythia*, *P. gingivalis* and periodontal pockets ≥5 mm (Mann–Whitney test, $p = 0.032$). *F. nucleatum* and *P. intermedia* were more frequently observed in patients displaying generalized gingival bleeding, but with no statistically significant association.

4. Discussion

P. gingivalis was the most prevalent bacterium in the atheromas. It was found in 50% of the atheroma samples and 75% of periodontal samples from patients with periodontitis. In 11 (39.3%) atheromatous samples, bacterial associations were detected, particularly involving *P. gingivalis* and other gram-negative organisms, as shown in Table 3. The associations between *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*, *E. faecalis* or other bacterium was also observed, in accordance with data previously reported [13,14]. Microbial association between *P. gingivalis*, *P. nigrescens* and *E. faecalis* was also detect in an atheroma surgically removed from a periodontally healthy patient. Our results are supported by previous study that confirms *P. gingivalis* as the most prevalent periodontal pathogen in atheromas [15] and this oral bacterium and others may contribute to the development of vascular diseases.

Table 3
Bacterial associations in 30 atheromatous plaques obtained from coronary arteries.

Microbial combinations	Prevalence No. (%)
Periodontitis patients ($N = 28$)	
Pg	6 (21.4)
Pg + *Pi*	3 (10.7)
Ef + *Pg*	3 (10.7)
Pn	3 (10.7)
Aa + *Pg*	1 (3.6)
Pe + *Pn*	1 (3.6)
Cr + *Pi*	1 (3.6)
Aa + *Pg* + *Pi*	1 (3.6)
Cr + *Ef* + *Td*	1 (3.6)
Tf	2 (7.1)
Polymicrobial infections	11 (39.3)
Periodontally healthy subjects ($N = 2$)	
Ef + *Pg* + *Pn*	1 (50.0)

Aa: *A. actinomycetemcomitans*; *Cr*: *C. rectus*; *Ef*: *E. faecalis*; *Pe*: *P. endodontalis*; *Pg*: *P. gingivalis*; *Pi*: *P. intermedia*; *Pn*: *P. nigrescens*; *Tf*: *T. forsythia*; *Td*: *T. denticola*.

The presence of *A. actinomycetemcomitans* has been associated to the aggressive periodontitis and various extra-oral infections, but in the present study, this bacterium was observed in two (7.1%) clinical samples from coronaries and in seven (25%) periodontal samples. Data showing the presence of this bacterium in atheromatous tissues has been observed varying from 16.6% to 26.4% [13,14], contrasting with the results presented in this study (Table 2). Surprisingly, *E. faecalis* and *P. endodontalis* were found in 4 (14.3%) and 1 (3.6%) of the atheromatous plaques, respectively, but they were more frequent in subgingival samples of patients with periodontitis. They are important bacteria in the root canal infections, but apparently no patient showed endodontic infections.

Studies referring to periodontal pathogens detected in atherosclerotic plaques from patients with periodontitis and healthy blood vessels undergoing coronary artery have been observed [16], but the role of a possible invasion of the vascular tissue by periodontal pathogens in the development and progression of atherosclerosis still remains questionable. The deepest atheroma plaques present a low oxygen tension providing conditions for the growth of anaerobes and probably periodontal pathogens play an important role in the atheroma pathogenesis and progression of cardiovascular disease [17]. Moreover, it is hard to establish if bacteria are resident or transitory, which determine the differences in detection rates observed in different studies [18]. Although, data in literature show discrepancies on the presence of periodontopathic bacteria in atheromatous plaques and it may be explained by differences in the subgingival microbial species, host immune response, ethnic, and socio-economic level of the population evaluated [19]. The presence of bacteria in atheroma samples might be explained as an intermittent stage of these microorganisms in bacteremia, but with real possibility to reach and to colonize the arteries [20]. In conclusion, it is possible that recognized periodontal pathogens present in subgingival oral biofilm may find a way to reach arteries,

and *P. gingivalis*, and other oral and non-oral bacteria play a direct or indirect role in the atheromatous plaques formation.

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