Journal of Medical Microbiology (2009), **58**, 1568–1575 DOI 10.1099/jmm.0.013383-0

Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries

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Oral pathogens, including periodontopathic bacteria, are thought to be aetiological factors in the development of cardiovascular disease. In this study, the presence of *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum–periodonticum–simiae* group, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *Tannerella forsythia* in atheromatous plaques from coronary arteries was determined by real-time PCR. Forty-four patients displaying cardiovascular disease were submitted to periodontal examination and endarterectomy of coronary arteries. Approximately 60–100 mg atherosclerotic tissue was removed surgically and DNA was obtained. Quantitative detection of periodontopathic bacteria was performed using universal and species-specific TaqMan probe/primer sets. Total bacterial and periodontopathic bacterial DNA were found in 94.9 and 92.3 %, respectively, of the atheromatous plaques from periodontitis patients, and in 80.0 and 20.0 %, respectively, of atherosclerotic tissues from periodontally healthy subjects. All periodontal bacteria except for the *F. nucleatum–periodonticum–simiae* group were detected, and their DNA represented 47.3 % of the total bacterial DNA obtained from periodontitis patients. *Porphyromonas gingivalis*, *A. actinomycetemcomitans* and *Prevotella intermedia* were detected most often. The presence of two or more periodontal species could be observed in 64.1 % of the samples. In addition, even in samples in which a single periodontal species was detected, additional unidentified microbial DNA could be observed. The significant number of periodontopathic bacterial DNA species in atherosclerotic tissue samples from patients with periodontitis suggests that the presence of these micro-organisms in coronary lesions is not coincidental and that they may in fact contribute to the development of vascular diseases.

Received 3 June 2009
Accepted 6 August 2009

INTRODUCTION

Atherosclerosis has been defined as a progressive process that causes focal thickening of large- to medium-sized muscular and large elastic arteries (Pucar *et al.*, 2007). In addition, several risk factors have been associated with its progression, such as hypertension, hypercholesterolaemia, diabetes, marked obesity, smoking, physical inactivity and chronic bacterial infection (Taylor-Robinson *et al.*, 2002; Pucar *et al.*, 2007). The development of atheromatous plaques seems to be relevant to cardiovascular disease as a result of endothelial cell damage and by maintenance of the inflammatory reactions in the walls of blood vessels (Libby *et al.*, 2002).

Studies have demonstrated a close association between cardiovascular disease and periodontitis, and oral hygiene and periodontal status are closely related to the occurrence of heart attacks (Kozarov *et al.*, 2006; Aimetti *et al.*, 2007; Johansson *et al.*, 2008). A relationship between periodontal status and cardiovascular diseases has been suggested, where the periodontium represents the source of inflammatory mediators, in addition to micro-organisms systemically disseminated by the blood flow (Li *et al.*, 2000).

Considerable evidence supports a plausible set of mechanisms by which periodontopathic bacteria may directly or indirectly contribute to cardiovascular disease, such as blood platelet aggregation, enhanced low-density cholesterol and lipoprotein deposition in the artery walls, invasion of cardiac and carotid endothelium, and the high level of inflammatory mediators in the circulation and tissues (Dorn *et al.*, 1999; Sharma *et al.*, 2000; Libby *et al.*, 2002). However, evidence that periodontal infections contribute to or are decisive factors in the development of atherosclerotic plaques is circumstantial, and an epidemiological association is not proof of a causal link between pathogens and cardiovascular disease, although bacterial presence at the diseased site is one of the

requirements to determine a causal relationship (Cairo *et al.*, 2008).

As a result of the high sensitivity of PCR and other molecular methods, the presence of micro-organisms inadvertently introduced into the blood flow or as a result of accidental contamination during endarterectomy may be confused with the presence of these pathogens within atheromatous plaques. However, with the advent of real-time PCR, it is possible to quantify the microbial DNA in order to differentiate the transient presence of micro-organisms colonizing and infecting the vascular walls and atheromatous lesions.

In recent years, studies have implicated *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Prevotella nigrescens*, *Porphyromonas gingivalis* and *Tannerella forsythia* in connective tissue attachment loss and periodontal inflammation (Ebersole *et al.*, 2008; Herrera *et al.*, 2008), whilst *Fusobacterium nucleatum* represents a bridge between the first and the late colonizers of oral subgingival biofilms (Kolenbrander, 2000). In this study, the presence of these micro-organisms in atheromatous plaques obtained from coronary arteries from patients with chronic periodontitis and from periodontally healthy subjects was determined.

METHODS

Patients. Forty-four adult patients (35 males and 9 females), from 36 to 82 years old (mean age 60 ± 11.3 years), displaying cardiovascular disease and seen at the Evangelic Hospital of Londrina (Londrina, Paraná, Brazil) and Hospital de São José do Rio Preto (São José do Rio Preto, SP, Brazil), were evaluated. Clinical samples were collected from March 2005 to December 2007. Patients fulfilling the inclusion criteria were informed of the study and signed an informed consent form that was approved by the Ethics Committee in Research of the University of São Paulo (no. 270/02).

Initially, before endarterectomy of coronary arteries, all patients underwent a clinical interview in order to obtain information about their identification, age, disease history, and medical and familial histories. Patients were submitted to a complete periodontal examination by a single periodontist, collecting data on tooth loss, plaque index (full-mouth plaque score), bleeding on probing (full-mouth bleeding score), probing depth, gingival recession and clinical attachment level at six sites (mesial, mid- and distal sites of oral and facial surfaces) per tooth, excluding third molars, using a manual periodontal probe (PCP UNC-15; Hu-Friedy).

The periodontal conditions of the patients are presented in Table 1: 39 patients displayed generalized chronic periodontitis with attachment loss exceeding 5 mm in ⩾30 % of the periodontal sites, whilst five patients were periodontally healthy (Tonetti & Claffey, 2005). Exclusion criteria included self-medication history, diabetes, autoimmune disease or other systemic pathology, and any periodontal or antibiotic therapy during the last 3 months.

All patients submitted to endarterectomy presented diffuse atherosclerotic disease and multiple stenoses with distal and diffuse involvement with reversible ischaemia, documented by scintigraphy and analysis of echocardiography, under conditions considered inoperable by conventional methods, or advanced atherosclerotic disease with viable myocardium.

Table 1. Clinical parameters at baseline in patients with chronic periodontitis and periodontally healthy subjects submitted to endarterectomy of coronary arteries

Values are means ± SD.

Clinical parameter	Periodontitis patients (n=39)	Periodontally healthy subjects (n=5)
Full-mouth plaque score (%)	60.5 ± 15.4	51.5 ± 12.5
Full-mouth bleeding score (%)	61.0 ± 22.5	17.58 ± 13.4
Probing depth (mm)	5.4 ± 1.4	1.55 ± 0.21
Clinical attachment level (mm)	5.7 ± 2.8	1.75 ± 0.22
Number of teeth	13.5 ± 5.7	20.5 ± 4.5

Atheromatous plaque sample collection. Atheromatous plaques from coronary arteries were removed surgically and placed in vials containing 10 ml sterile DNA-free saline solution and stored at −20 °C. Only atheromatous plaques were removed and processed in order to avoid damage to vascular walls. A sagittal section was made through the middle of the atherosclerotic plaque. Approximately 100 mg tissue was placed in a vial containing 5 ml RNAlater (Ambion; Applied Biosystems) for DNA extraction by using a Charge Switch gDNA Mini Tissue kit (Invitrogen) according to the manufacturer's instructions. DNA was stored at −20 °C until amplification by real-time PCR. Purified genomic DNAs from *A. actinomycetemcomitans* ATCC 29523, *F. nucleatum* ATCC 10953, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611 and *T. forsythia* ATCC 43037 were used as positive controls. DNA concentrations were determined spectrophotometrically by measuring the A_{260} (model DU-640; Beckman Instruments).

Quantitative analysis by real-time-PCR. Real-time PCR assays were carried out using a Rotor Gene 6000 (Corbett Life Science). For amplification reactions, duplicate samples were routinely used and assays were performed in a total volume of 25 µl containing 12.5 µl 2× Taqman Universal Master Mix (Applied Biosystems), 0.2 µl each forward and reverse primer (final concentration 200 nM each), 0.1 µl Taqman probe (final concentration 100 nM), 2 µl template DNA solution and an appropriate volume of sterilized DNase- and RNase-free water. Amplification reactions were performed in a thermocycler programmed as follows. For detection of total bacterial DNA for *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and *F. nucleatum*, an initial denaturation at 95 °C for 10 min was followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. For *T. forsythia*, an initial denaturation at 95 °C for 10 min was followed by 45 cycles at 95 °C for 15 s and 55 °C for 1 min. The negative control was a PCR TaqMan Master Mix without DNA. The primer/probe sets used are shown in Table 2.

The total number of bacterial cells was also determined using the TaqMan PCR procedure with a universal primer/probe set. The standard curve was analysed for each evaluated bacterium and using the universal primer/probe set against a serial dilution of each bacterial DNA corresponding to 10^2–10^7 cells, and showed an error value range of 0.0288–0.0695. A sample was considered positive for a target micro-organism when the fluorescence emitted by the sample was at least 50 % higher than the limit of detection previously established for the micro-organism and 50 % above the background fluorescence.

Table 2. Specific primers and probes used in the real-time PCR

Micro-organism	Primer/TaqMan probe sequence (5′→3′)	Reference
A. actinomycetemcomitans		Maeda *et al.* (2003)
Forward	CTTACCTACTCTTGACATCCGAA	
Reverse	ATGCAGCACCTGTCTCAAAGC	
Probe	FAM-AGAACTCAGAGATGGGTTTGTGCCTTAG-TAMRA	
F. nucleatum–periodonticum–simiae group		Suzuki *et al.* (2005)
Forward	CGCAGAAGGTGAAAGTCCTGTAT	
Reverse	TGGTCCTCACTGATTCACACAGA	
Probe	FAM-ACTTTGCTCCCAAGTAACATGGAACACGAG-TAMRA	
Porphyromonas gingivalis		Nonnenmacher *et al.* (2004)
Forward	TGCAACTTGCCTTACAGAGGG	
Reverse	ACTCGTATCGCCCGTTATTC	
Probe	FAM-AGCTGTAAGATAGGCATGCGTCCCATTAGCTA-TAMRA	
Prevotella intermedia		Nonnenmacher *et al.* (2004)
Forward	CCACATATGGCATCTGACGTG	
Reverse	TCAATCTGCACGCTACTTGG	
Probe	FAM-ACCAAAGATTCTACGGTGGAGGATGGG-TAMRA	
Prevotella nigrescens		Kuboniwa *et al.* (2004)
Forward	CCGTTGAAAGACGGCCTAA	
Reverse	CCCATCCCTTACCGGRA	
Probe	FAM-CCCGATGTGTTTCATTGACGGCATC-TAMRA	
T. forsythia		Kuboniwa *et al.* (2004)
Forward	AGCGATGGTAGCAATACCTGTC	
Reverse	TTCGCCGGGTTATCCCTC	
Probe	FAM-CCTCTGTATACGCCATTGTAGCACGTGTGT-TAMRA	
Universal		Nonnenmacher *et al.* (2004)
Forward	TGGAGCATGTGGTTTAATTCGA	
Reverse	TGCGGGACTTAACCCAACA	
Probe	FAM-CACGAGCTGACGACA(AG)CCATGCA-TAMRA	

***fimA* genotyping for *Porphyromonas gingivalis*.** Samples of atheromatous plaques were assayed to detect *fimA* genotypes for *Porphyromonas gingivalis*. The tests were performed basically as described previously by Amano *et al.* (1999) and Nakagawa *et al.* (2000, 2002b). Table 3 lists the PCR primers used. A ubiquitous primer set that matches almost all bacterial 16S rRNA genes was used as a positive control, and *Porphyromonas gingivalis* species-specific primers (16S rRNA gene-specific) were used for *fimA* typing. All primers were custom-made by Invitrogen.

PCR amplification was performed in a volume of 25 μl containing 1 × PCR/Mg^{2+} buffer (Boehring Mannheim), 0.2 mM each dNTP (Pharmacia Biotech), 0.5 U *Taq* DNA polymerase (Invitrogen), 0.4 μM each primer pair (Invitrogen) and 10 ng template.

Table 3. Primer sets used for *Porphyromonas gingivalis fimA* genotyping

Primer set	Sequence (5′→3′)	Amplicon size (bp)	Reference
P. gingivalis 16S rRNA gene	TGTAGATGACTGATGGTGAAAACC ACGTCATCCCCACCTTCCTC	197	Amano *et al.* (1999)
Type I *fimA*	CTGTGTGTTTATGGCAAACTTC AACCCCGCTCCCTGTATTCCGA	392	Amano *et al.* (1999)
Type Ib *fimA*	CAGCAGAGCCAAAAACAATCG TGTCAGATAATTAGCGTCTGC	271	Nakagawa *et al.* (2002b)
Type II *fimA*	ACAACTATACTTATGACAATGG AACCCCGCTCCCTGTATTCCGA	257	Amano *et al.* (1999)
Type III *fimA*	ATTACACCTACACAGGTGAGGC AACCCCGCTCCCTGTATTCCGA	247	Amano *et al.* (1999)
Type IV *fimA*	CTATTCAGGTGCTATTACCCAA AACCCCGCTCCCTGTATTCCGA	251	Amano *et al.* (1999)
Type V *fimA*	AACAACAGTCTCCTTGACAGTG TATTGGGGGTCGAACGTTACTGTC	462	Nakagawa *et al.* (2002b)

Amplification was performed in a DNA thermal cycler (GeneAmp PCR System 9700; Perkin Elmer) at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplification products were analysed by electrophoresis in 1 % agarose gels in 1× TBE buffer [1 M Tris/HCl (pH 8.4), 0.9 M boric acid, 0.01 M EDTA; Invitrogen], stained with 0.5 mg ethidium bromide ml^{-1}.

Statistical analysis. Mean values ± SD were calculated for each bacterial species and for total eubacteria. Statistical analyses were performed using the software SPSS, version 13. Differences between clinical and microbiological qualitative parameters were evaluated using a Mann–Whitney, χ^2 or Fisher's exact test. For evaluation of the quantitative microbial parameters, data obtained from patients harbouring these bacteria were also computed. A difference of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

By using the universal and specific primer/probe sets, total bacterial DNA and periodontopathic bacteria DNA were detected in 94.9 and 92.3 %, respectively, of atherosclerotic samples from patients with periodontitis. DNA of the targeted periodontal bacteria represented a mean of 47.3 % of the total bacterial DNA observed in atheromas, and *Porphyromonas gingivalis* DNA comprised 18.8 % of the microbial DNA in these samples (Table 4), whilst fusobacteria were not detected. Bacterial DNA was found in 80 % of atheromatous plaques recovered from periodontally healthy subjects, and *Porphyromonas gingivalis* was the only targeted micro-organism detected in these atheroma samples from healthy subjects (20 %).

Prevotella intermedia (59.0 %), *Porphyromonas gingivalis* (53.8 %) and *A. actinomycetemcomitans* (46.2 %) were the most prevalent bacteria, followed by *T. forsythia* (25.6 %) and *Prevotella nigrescens* (17.9 %), in the atheromas from patients with periodontitis (Table 4). Moreover, in most of the samples, the presence of DNA from more than one periodontal pathogen was observed, particularly *A. actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* (Table 5). The concomitant detection of two or more species was observed in 64.1 % of the clinical samples from periodontitis patients, whilst the presence of a single species was detected in 28.2 % of the samples.

The presence of these periodontopathic bacteria in atherosclerotic plaques was not related to age ($P=0.188$), sex ($P=0.633$), number of teeth ($P=0.352$) or tobacco use ($P=0.082$) (all using Fisher's exact test). The presence of DNA of the targeted micro-organisms (mean ± SD) is shown in Fig. 1. In our study, *Porphyromonas gingivalis* was detected in the highest numbers, followed by *Prevotella intermedia* and *A. actinomycetemcomitans*. The DNA levels of *T. forsythia* and *Prevotella nigrescens* were similar ($P=0.171$).

The presence of periodontopathic bacteria in atheromatous plaques from coronary arteries is associated with the concept that periodontal bacteria such as *A. actinomycetemcomitans* and especially *Porphyromonas gingivalis* are able to invade endothelial cells and induce a chronic vascular inflammation (Yuan *et al.*, 2008). Moreover, *A. actinomycetemcomitans*, *F. nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens* and other oral anaerobes and microaerophiles can degrade immunoglobulin, preventing the action of the complement system, and can produce some toxic components such as endotoxins and exotoxins (Fives-Taylor *et al.*, 1999; Brook, 2007). These factors may help micro-organisms to maintain their viability during bacteraemia, which occurs frequently as a result of surgical procedures, tooth brushing and other dental procedures (Dorn *et al.*, 1999; Daly *et al.*, 2001).

Studies have associated the presence of *Porphyromonas gingivalis*, *A. actinomycetemcomitans*, *T. forsythia* and *Prevotella intermedia* DNA in atheromatous plaques with the severity of periodontal disease, increasing with the age of the patients (Haraszthy *et al.*, 2000; Pucar *et al.*, 2007).

Table 4. Bacterial distribution in atheromatous plaques from 44 patients submitted to endarterectomy of coronary arteries

Micro-organisms	Prevalence [*n* (%)]	Total bacterial DNA (%)*
Periodontitis patients ($n=39$)		
A. actinomycetemcomitans	18 (46.2)	11.3
Porphyromonas gingivalis	21 (53.8)	18.8
Prevotella intermedia	23 (59.0)	13.2
Prevotella nigrescens	7 (17.9)	1.6
T. forsythia	10 (25.6)	2.4
F. nucleatum–periodonticum–simiae group	0 (0.0)	0.0
Total periodontal bacteria	36 (92.3)	47.3
Total bacterial DNA	37 (94.9)	100
Periodontally healthy subjects ($n=5$)		
Porphyromonas gingivalis	1 (20.0)	7.2
Total bacterial DNA	4 (80.0)	100

*Mean percentage of the target bacterial DNA compared with the total DNA from samples.

Table 5. Bacterial associations in 44 atheromatous plaques obtained from coronary arteries

Microbial combinations	Prevalence [*n* (%)]	% total bacterial DNA*
Periodontitis patients (*n*=39)		
A. actinomycetemcomitans+*Porphyromonas gingivalis*+*Prevotella intermedia*+*Prevotella nigrescens*+*T. forsythia*	2 (5.1)	58.7
Porphyromonas gingivalis+*Prevotella intermedia*+*Prevotella nigrescens*+*T. forsythia*	1 (2.6)	61.0
A. actinomycetemcomitans+*Porphyromonas gingivalis*+*Prevotella intermedia*	4 (10.3)	54.0
A. actinomycetemcomitans+*Prevotella intermedia*+*Prevotella nigrescens*	4 (10.3)	48.6
A. actinomycetemcomitans+*Porphyromonas gingivalis*+*T. forsythia*	2 (5.1)	49.0
Porphyromonas gingivalis+*Prevotella intermedia*	5 (12.8)	15.5
Prevotella intermedia+*T. forsythia*	3 (7.7)	9.3
A. actinomycetemcomitans+*T. forsythia*	2 (5.1)	7.1
A. actinomycetemcomitans+*Porphyromonas gingivalis*	2 (5.1)	23.0
A. actinomycetemcomitans	2 (5.1)	9.01
Prevotella intermedia	4 (10.3)	42.1
Porphyromonas gingivalis	5 (12.8)	24.3
Periodontally healthy subjects (*n*=5)		
Porphyromonas gingivalis	1 (20.0)	7.2

*Percentage of bacterial DNA obtained from each target species compared with the total DNA from clinical samples.

In the present study, the prevalence of bacterial DNA in atheromas was higher than that reported by Ishihara *et al.* (2004) and Padilla *et al.* (2006), but similar results were reported by Lehtiniemi *et al.* (2005) and Kozarov *et al.* (2006) using a quantitative SYBR Green PCR assay. Bacterial DNA was observed in 94.9 % of atheromas from periodontitis patients and in 80.0 % of atheromas from periodontally healthy subjects. This result is in agreement with the results of Fiehn *et al.* (2005) and Aimetti *et al.* (2007), who reported the presence of bacterial DNA in 100 and 94 %, respectively, of the atheromas analysed. Moreover, the target periodontopathic bacterial DNA evaluated here represented 47.3 % of the total bacterial DNA found in atheromatous samples from periodontitis patients and 7.2 % of the DNA detected in atheromas from periodontally healthy subjects.

Due to the sensitivity of PCR for detecting microbial DNA, doubts have been raised about sample contamination as a result of bacteraemia or handling of samples (Sanz *et al.*, 2004). However, because of the high level of microbial DNA observed in most of the atheromatous plaques, it is

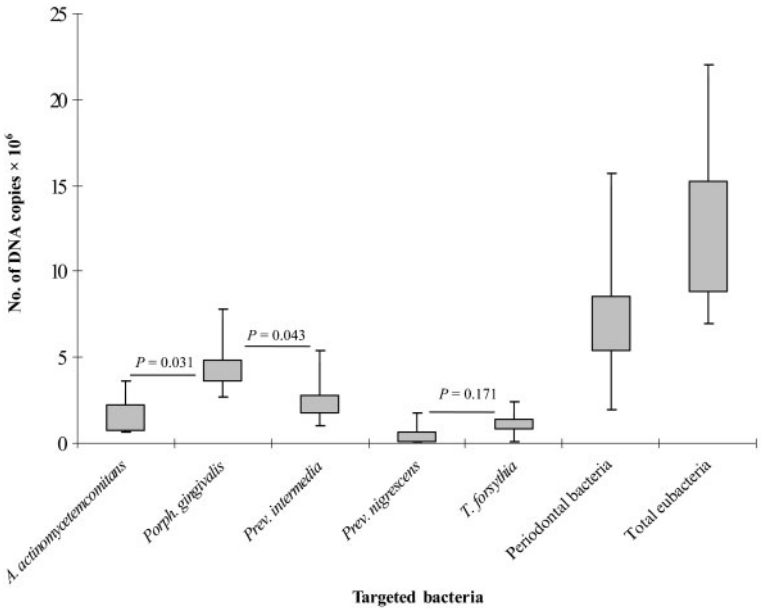

Fig. 1. Quantitative analysis (box plot) of the presence of periodontopathic bacteria in atheromas from coronary arteries of patients with chronic periodontitis.

unlikely that these patients suffered accidental contamination; instead, it suggests a stable and lasting microbial colonization of atheromas. It is well known that Gram-negative anaerobes can evade the immune system and that they possess proteolytic activity associated with acute and suppurative infections, particularly abscesses and cellulitis (Brook, 2008), but it has not been observed in tissue removed during endarterectomy.

The high prevalence of *Porphyromonas gingivalis*, *Prevotella intermedia* and *A. actinomycetemcomitans* may reflect the periodontal condition of the patients, as most of the infected atheromas were obtained from chronic periodontitis patients and these micro-organisms are highly prevalent in patients with periodontal pockets, subgingival plaque, insertion loss, periodontal bone loss and inflammation (Ebersole *et al.*, 2008; Herrera *et al.*, 2008).

Genotyping of the gene *fimA* was performed for the 21 atheromatous samples that were positive for *Porphyromonas gingivalis*, and this gene was detected in the atheromas from these patients. The genotype *fimA* II was detected in 11 (52.4 %) of the atheromatous plaques from patients with periodontitis and in only one clinical sample obtained from a periodontally healthy subject, whereas *fimA* IV was detected in six (28.6 %) of the atheromas. Interestingly, genotype *fimA* V, which is considered to be relatively rare in patients with chronic periodontitis (Nakagawa *et al.*, 2002b; Miura *et al.*, 2005; Enersen *et al.*, 2008), was observed in four (19.0 %) of the atheromatous plaques. In addition, a recent study suggested the involvement of genotypes *fimA* II and *fimA* IV in the initiation and progression of cardiovascular diseases (Nakano *et al.*, 2008). Moreover, both *fimA* genotypes also represent those most commonly observed in periodontitis (Miura *et al.*, 2005; Zhao *et al.*, 2007; Enersen *et al.*, 2008). Theoretically, the genotypes of the *fimA* gene show different responses to bacterial virulence and to periodontal treatment. As *Porphyromonas gingivalis* genotype *fimA* II shows an increased capacity to adhere to and invade human epithelial cells (Nakagawa *et al.*, 2002a) and to colonize the gingival crevice (van der Ploeg *et al.*, 2004), the significant occurrence of this genotype in atheromatous plaques may reflect these differences in virulence, although this hypothesis remains to be evaluated.

The predominance of these three micro-organisms in the clinical samples may be due to their ability to persist in vascular tissue in which latent intracellular bacteria are transformed to a viable state (Li *et al.*, 2008). In addition, periodontal patients harbouring *Porphyromonas gingivalis* have shown higher levels of lipids, low-density lipoprotein and total cholesterol in the bloodstream, which seem to be associated with the development of atheromatous plaques (Cutler *et al.*, 1999; Cairo *et al.*, 2008).

The development of atheromas induces fibrosis, as well as cholesterol and lipoprotein deposition, producing a reduced redox potential suitable for the development of anaerobic bacteria such as *Porphyromonas gingivalis*, *T. forsythia* and *Prevotella intermedia*, which are considered to be the most prevalent oral pathogens in atherosclerotic plaques (Fiehn *et al.*, 2005). On the other hand, the high prevalence of Gram-negative oral bacteria in atherosclerotic tissues may induce the secretion of several cytokines associated with the development of cardiovascular diseases by their LPS (Fiehn *et al.*, 2005).

Studies in elderly patients with chronic periodontitis have shown high detection rates of periodontopathic bacteria in atheromatous plaques; in contrast, in young patients, these bacteria are rarely present (Kozarov *et al.*, 2006). The difference observed in the literature on the detection of periodontopathic bacteria from atherosclerotic plaques may be explained by the methodologies employed, periodontal status, bacterial biofilm composition, socioeconomic and educational levels, and the ethnic characteristics of the populations examined (Fiehn *et al.*, 2005).

It is important to note that the presence of DNA from more than one periodontal species in atheromas may be relevant in the progression of atherosclerosis, as immunological data support the suggestion that association of three or more different micro-organisms is epidemiologically implicated in the pathogenesis of atherosclerosis and may lead to myocardial infarction (Haheim *et al.*, 2008). Our results suggest that microbial infections in the coronary arteries are essentially mixed, as 64.1 % of the clinical samples from periodontitis patients showed the presence of at least two different periodontopathic bacteria in the atherosclerotic samples.

As most infections associated with oral micro-organisms are mixed, with different species establishing ecological relationships with each other and with the host, it is possible that the mixed contamination detected in 64.1 % of atheromatous plaques from patients with periodontitis may reflect the complex ecological interactions that these organisms maintain in the oral cavity (Kolenbrander, 2000; Brook, 2007).

On the other hand, asymptomatic bacteraemia produced by periodontopathic bacteria may accelerate the progression of atheromatous plaques (Ishihara *et al.*, 2004). Thus, taken together with our results, this suggests that periodontal disease-associated or non-associated bacteria reach the bloodstream, playing a direct or indirect role in the pathogenesis of cardiovascular diseases. The results shown here reinforce the importance of periodontal bacteria as a possible contributing factor in the development of cardiovascular diseases. Knowledge of the micro-organisms present in atheromas from patients with chronic periodontitis is relevant in the prevention and treatment of cardiovascular infections that appear to be produced, in part, by these oral periodontopathic bacteria.

ACKNOWLEDGEMENTS

This study was supported by grants from the Fundação do Amparo à Pesquisa do Estado de São Paulo (FAPESP) Proc. No. 04/03199-3 and 07/51016-3.

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