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Original article

Detection of bacterial DNA in atheromatous plaques by quantitative PCR

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Abstract

This is the first study to analyze atheromatous plaques for the presence of bacterial DNA from ten species, including periodontal species and *Chlamydia pneumoniae*. We examined 129 samples of DNA extracted from atheromas from 29 individuals for the presence of bacterial 16S rDNA sequences from ten different species: *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* (*A.a.*), *Tannerella forsythensis*, *Eikenella corrodens*, *Prevotella intermedia*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Treponema denticola* and *C. pneumoniae*. All determinations were made using real-time quantitative polymerase chain reaction (PCR) methods employing SYBR®Green. Species from the *Bacteroides* family were found in about 17% of the young but ~80% in elderly patients. Almost half of the samples contained DNA from *A. a.* and *C. pneumoniae*, although the proportion of the latter was minimal. *S. aureus* and *S. epidermidis* were found with the lowest frequency, 5 and 10%, respectively. *S. mutans* was found in ~20% of the samples. The proportions of each bacterial species were calculated relative to the total amount of prokaryotic DNA. The data support our previous findings of an association between periodontal organisms and vascular inflammation. We conclude that DNA from oral infectious agents is commonly found in atheromas from young but especially from elderly subjects, and that the contribution of *C. pneumoniae* to the inflammation may be minimal.

Keywords: Atheroma; Periodontitis; Bacteria

1. Introduction

Epidemiological studies have revealed that a high proportion of patients with cardiovascular disease (CVD) have periodontal disease or are edentulous. A prospective cohort study of 9760 American adults linked CVD and periodontal disease, concluding that ''dental disease is associated with increased risk of CVD, particularly in young men'' [1]. According to a study of 1372 native Americans, individuals with periodontal disease were 2.7 times as likely to suffer a heart attack than were those with better oral hygiene. Periodontal disease was a more important risk factor than high blood pressure, and second only to having long-term diabetes in this study [2]. The cell proliferation and inflammatory character of the atheromatous lesion may well be due to an infectious component [3]. Measurement of C-reactive protein (CRP) concentration, a marker for systemic inflammation, in the plasma of 543 healthy men demonstrated that the higher the CRP content, the higher the incidence of myocardial infarction or ischemic stroke [4]. This conclusion was supported by a prospective cohort study of 14,719 initially healthy

Abbreviations: CRP, C-reactive protein; CVD, Cardiovascular disease; PDAY, Pathobiological Determinants of Atherosclerosis in Youth; PCR, Polymerase chain reaction.

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women [5]. Indeed, the presence of the periodontal pathogens *P. gingivalis*, *P. intermedia*, *Campylobacter recta*, and *Bacteroides fragilis* in subgingival samples was positively associated with elevated CRP levels ($P = 0.029$) [6]. Since one of the hallmarks of periodontal disease is inflammation, it is possible that periodontal pathogens may be involved in this increase in CRP as a source of chronic or sustained inflammation. However, the evidence that periodontal infection is a contributing factor is somewhat circumstantial since epidemiological association is not proof of a causal link between the pathogen and CVD. Evidence of bacterial presence at the diseased site is one of the requirements to determine a causal relationship.

The concept that the periodontopathogenic bacterium, *P. gingivalis*, invades and degrades oral epithelium is now well accepted. *P. gingivalis* is considered an etiologic agent of adult forms of periodontal disease [7]. *P. gingivalis* invades human epithelial cells [8], likely to avoid the immune defense and to find its privileged niche. Oral epithelia are the primary site for *P. gingivalis* infection but this bacterial species can enter the circulation following tooth brushing and other dental procedures [9]. This work relates to the systemic implications of the disease, in particular the presence of the bacterium in endothelial tissues. We know that (1) there is a large body of evidence that *P. gingivalis*, an asaccharolytic Gram-negative rod, has developed an elaborate proteolytic system composed of several surface-located or secreted enzymes (gingipains), which apparently serve a role to provide bacteria with nutrients in the form of small peptides and amino acids leading to tissue destruction [10,11]. These proteinases are also implicated in the degradation of collagen [12], fibronectin and fibronectin integrin receptor [13]. (2) The effect of two arginine-specific cysteine proteinases (gingipain Rs) from *P. gingivalis* supports the hypothesis that induction of blood coagulation by bacterial proteinases may contribute to the pathogenesis of disseminated intravascular coagulation in sepsis. Gingipains R are the first-reported activators of factor IX of bacterial origin. By this effect they could be involved in the production of thrombin and the activation of platelets in the clotting pathway in blood coagulation [14,15]. (3) In addition, there is evidence that *P. gingivalis* may be able to invade the deeper structures of connective tissues via a paracellular pathway by degrading epithelial cell–cell junction complexes, thus allowing the spread of the bacterium [16,17]. (4) Oral organisms including *P. gingivalis* have been immunolocalized within unstable plaque regions and associated with plaque ulceration, thrombosis, and apoptosis in vascular cells [18].

To determine the invasive ability of oral bacteria in vascular tissue, an extensive study of *P. gingivalis* invasion of human coronary artery endothelial cells was performed [19]. Moreover, it was determined that the ability of various *P. gingivalis* strains to invade human vascular cell lines varies greatly [20]. The strains tested exhibited invasion efficiencies differing by orders of magnitude. Invasiveness and cytotoxicity have been reported for *A.a.* as well [21,22]. Further, DNA from *P. gingivalis*, *A.a.*, *Tannerella forsythensis* and *Prevotella intermedia* has previously been identified in atheromatous plaques [23–25]. In addition, *P. gingivalis*-specific T-cell lines have been established from atherosclerotic lesions [26,27]. A correlation has also been made between the detection rate in atheromas of DNA of some periopathogens including *P. gingivalis* and their presence in subgingival plaque [28]. Finally, we demonstrated the presence of live periodontal bacteria in atheromatous tissue [29].

In summation, present knowledge is that (1) bacterial infectious agents are associated with periodontitis, the most prevalent chronic inflammatory infectious disease; (2) epidemiological and seroepidemiological evidence has emerged linking infectious diseases, including periodontal disease with CVD; (3) periodontal species invade human endothelial cells.

Based on the accumulated evidence, it is thus reasonable to focus on the presence of oral pathogens as a likely contributor to vascular inflammatory lesion development. For this reason, the objective of our study was to quantitatively determine the presence of a number of oral bacteria in 129 DNA samples extracted from aortic lesions of 29 individuals. To assess the contribution of another pathogen, *C. pneumoniae* that is suggested by some studies to be associated with atherosclerotic lesions, we included it as well. The analysis was done for the following species: *P. gingivalis*, *A. actinomycetemcomitans*, *T. forsythensis*, *E. corrodens*, *P. intermedia*, *S. aureus*, *S. epidermidis*, *S. mutans*, *T. denticola*, and *C. pneumoniae*.

2. Materials and methods

2.1. Subjects

Two groups of individuals were used as a source of atheromatous tissues. Group A consisted of 120 samples from 20 young individuals (11 males and 9 females, six samples per individual, mean age 26.8 ± 8.2 years) and were obtained from Dr Gray Malcom of the Louisiana State University Pathobiological Determinants of Atherosclerosis in Youth (PDAY) program from forensic autopsies. A description of PDAY and the subject group is found in reference [30]. The second group of nine samples (group B) was from elderly individuals (mean age 66.9 ± 13.5 years) who had undergone endarterectomy and whose dentition condition has also been recorded. These samples were obtained from Dr J. Seeger, Department of Surgery, University of Florida. Only one out of the nine patients studied had a full dentition; all others had dentures (one is edentulous; see Table 1).

2.2. Atheroma collection and handling

Atheromatous tissue was collected under aseptic conditions and placed in 30 ml of pre-reduced transport medium immediately upon resection. The tissue was then was transported to the lab facility where a fraction of it was removed in a laminar-flow hood at aseptic conditions and used for DNA isolation. During the transfer of the atheromatous tissue, it was presumed that the remnants of the microcapillary blood from the tissue, estimated at 50 μg/g tissue in vivo, leaked into the medium. Consequently, the traces of blood in the sections of the tissue used for DNA isolation were minimal.

Table 1
Characteristics of group B subjects

Subject	Age	Sex	Smoker	Location*	Dentures	Current/past diagnoses
1	74	F	Y	Carotid*	Edentulous, none	CNS stroke/HBP
2	59	M	N (quit 4 years previous)	Carotid	Upper denture	Graft disruption/HBP, bypass
3	67	M	Y	Left carotid	Full dentition, none	Seizure/HBP, bypass
4	78	F	Y	Right ileac/ femoral	Partial denture	Endarterectomy, CAD
5	71	F	No data	Right carotid	Upper denture	Seizure/CAD, HBP, NIDDM
6	68	M	Y	Left carotid	Upper and lower dentures	Strokes
7	35	F	Y	Aortic	Upper denture	Ulceration, significant stenosis
8	70	M	Y	Aortic*	Upper and lower dentures	Abdominal aortic aneurysm/HBP
11	80	F	N	Femoral	Partial denture	Stroke/IDDM, CABG, stroke

Abbreviations: HBP, high blood pressure; CAD, coronary artery disease; NIDDM, non-insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; CABG, coronary artery bypass graft; CNS, central nervous system. *Indicates presence of calculi.

2.3. Atheroma DNA isolation and testing

Total DNA from the atheroma samples and genomic DNA from target bacteria from mid-log phase cultures were purified using a QIAmp Tissue Kit (Qiagen Inc., Valencia, CA) and the concentration determined by a DNA dye-binding assay (Pico-Green, Molecular Probes, Eugene, OR). The concentration of bacterial DNA for standards was adjusted to 10 μg/ml in water and then six additional serial dilutions (1:10) were made. Two replicate samples of each dilution (2 μl/sample) were then assayed as described above for the subject DNA. The sensitivity of each assay was determined to be the lowest dilution of DNA that produced a statistically significant ($p < 0.001$) average signal higher than the control (no template) using a *t*-test. For our calculations, we assumed that the average cell contained about 10 fg of genomic DNA and about seven copies of each 16S rRNA gene [31].

2.4. PCR primers and probes

Amplification primers were designed using Primer-Express™ software (Applied Biosystems, Foster City, CA). Briefly, sequences for the 16S rRNA genes of the organisms of interest were aligned and inspected for regions of conserved and variable sequences. Regions specific for target bacteria were selected and evaluated for Tm and several other characteristics using the PrimerExpress™ software. Sequences that satisfied these criteria were evaluated using BLAST [32] and/or Gapped BLAST [33] searches. Sequences with appropriate specificity were designated as probes and appropriate amplification primers were designed, again using the Primer-Express™ software (see Table 2).

Amplification primers designed by other laboratories were synthesized according to the sequences published by their authors and examined in the same manner (see references in Table 2). All primers were tested for specificity using 40–50 laboratory strains of bacteria.

2.5. Bacterial genomic DNA isolation

A. actinomycetemcomitans, *P. gingivalis*, *P. intermedia*, *S. mutans*, *S. aureus*, *S. epidermidis*, *T. denticola*, *E. corrodens*, *C. pneumoniae* and *T. forsythensis* were grown to mid-log phase according to their specific media and conditions (http://www.atcc.org). Bacterial genomic DNA was collected using the Wizard® Genomic DNA Isolation kit (Promega) according to the manufacturer's instructions. Once isolated, the genomic DNA was stored at −80 °C until used.

2.6. Quantitative PCR reaction

Five microliter aliquots of each sample were tested in triplicate using a quantitative PCR method [34] with SYBR®Green dye to detect the 16S rDNA amplicon accumulation. A SYBR®Green PCR Master Mix (Applied Biosystems) of all components except target DNA and primers was used

Table 2
Organism-specific primers

A. actinomycetemcomitans	ggcacgtaggcggacctt	accagggctaaagcccaatc	
T. forsythensis	gggtgagtaacgcgtatgtaacct	acccatccgcaaccaataaa	[34]
C. pneumoniae	catgtattgacaactgttagaaatacagc	caacacctcacggcacgag	
E. corrodens	gggaagaaaagggaagtgct	tcttcaggtaccgtcagcaaaa	
P. gingivalis	catagatatcacgaggaactccgatt	aaactgttagcaactaccgatgtgg	
P. intermedia	agattgacggccctatgggt	ccggtccttattcgaagggta	
S. epidermidis	tacacaccgcccgtcaca	cacccgaagccggtggagtaacc	[39]
S. mutans	agcgttgtccggatttattgg	agagcacactatggttgagcca	
S. aureus	tcggtacacgatattcttcac	actctcgtatgaccagcttc	[40]
T. denticola	cttccgcaatggacgaaagt	caacctttcggccttcttca	
Universal	ccatgaagtcggaatcgctagt	gcttgacgggcgtgtg	[34]

Primers without references were designed as part of the experimental protocol for this study (see Section 2).

throughout these studies. The components were combined and 23 μl of mix were dispensed into a 96-well assay plate. Duplicate samples of target DNA (2 μl) from plaque samples or dilutions of bacterial genomic DNA were then added to the wells. The plates were sealed with a clear plastic adhesive sheet, placed in an ABI 7700 Sequence Detector and cycled 40 times (94 °C, 15 s, 60 °C 1 min) after an initial denaturation at 94 °C, for 10 min. Data were collected during each run through optical cables over each well. The ABI 7700 software calculates a C_t value that is proportional to the number of gene copies in the original sample. The exact relationship was estimated using the genomic DNA standard curve. Proportions were calculated using the total bacterial numbers as determined by the PCR assay using the universal prokaryotic primer set, e.g., Proportion *P. gingivalis* = 100% × (fg *P. gingivalis* DNA/fg total prokaryotic DNA). Primers designed for the following bacteria were used: *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *S. mutans*, *S. aureus*, *S. epidermidis*, *T. denticola*, *E. corrodens*, *C. pneumoniae* and *T. forsythensis*.

2.7. Quantitative PCR standard curve

Genomic DNA from *P. gingivalis* was purified from mid-log phase cultures using a Wizard? Genomic DNA Isolation Kit (Promega), and the concentration determined by a DNA dye-binding assay (Pico-Green, Molecular Probes, Eugene, OR). DNA was demonstrated to be free of RNA by comparison of OD_{260} readings before and after treatment with DNase-free RNase. The concentration of DNA was adjusted to 2 mg/ml in water and then six additional serial dilutions (1:10) were made. Ten replicate samples of each dilution (5 μl/sample) were then assayed as described above for the subject DNA. The sensitivity of each assay was determined to be the lowest dilution of DNA that produced a statistically significant ($p < 0.001$) average signal higher than the no template control using a *t*-test.

2.8. Quantitative PCR

A SYBR®Green PCR Master Mix (Applied Biosystems) of all components except target DNA and primers was used throughout these studies. The components were combined and 23 μl of mix was dispensed into a 96-well assay plate. Duplicate samples of target DNA (2 μl) from plaque samples or dilutions of bacterial genomic DNA were added to the wells and cycled in an ABI 7700 Sequence Detector 40 times at the conditions described above. Data were collected during the run through optical cables over each well.

3. Results

One of the main objectives of this study was to determine if the DNA from the atheromas contained enough bacterial DNA to make quantitative measurements possible. Initial experiments with primers designed to amplify all 16S rDNA from bacterial sources verified that 1.5–2.2% of the total DNA in the samples was bacterial in origin. Then using these values as the denominator, we calculated the proportion of each species in each sample.

Samples were considered positive for a particular organism if the values were three standard deviations above the mean of the negative control. Data representing levels of bacterial DNA belonging to a total of ten species in the samples are presented as mean % of the total bacterial DNA in Tables 3 and 4. It is noteworthy that all organisms tested for were found in at least some of the subjects. The data are presented in aggregate on a subject basis as means of multiple samples per subject. Therefore, if at least one sample per subject is positive the subject is reported as positive for the organism.

The species representation as percent of the total bacterial DNA and the prevalence of bacteria in the specimens are represented in Fig. 1A and B, respectively.

We found between 15 (PI and TF, Table 3) and 89% (PG, Table 4) of the individual samples contain DNA from specific oral bacterial species using the Q-PCR assay. The Q-PCR detection of bacterial 16S rDNA from group B (the elderly group of patients) demonstrated the prevalence of periodontal species compared to others, including *C. pneumoniae*. Eight of the nine group B samples contain *P. gingivalis* DNA (89%) closely followed by *P. intermedia* (78%).

Quantitation of the bacterial DNA in the specimens gave us the opportunity to estimate the number of bacterial genomes per specimen, as represented in Table 5.

4. Discussion

In this work, we exploited the advantages offered by the quantitative PCR technology for a very sensitive and statistically significant determination of the number of bacterial genomes in the sample. We were able to obtain sufficient data from our samples to estimate not only the prevalence but also the proportions of each species, which will allow normalization of samples to overcome the inherent problem of

Table 3
Mean levels of bacterial DNA in 120 atheroma samples from group A (six from each of 20 young individuals)

Species	AA	PG	PI	SM	SA	SE	TD	EC	TF	CP
Mean %	5.82	6.10	0.36	2.90	0.25	0.13	2.35	1.84	0.47	1.13
% positive samples	40	18.3	15	22.5	5	10.8	19.1	17.5	15	44

Abbreviations: AA, *A. actinomycetemcomitans*, PG, *P. gingivalis*, PI, *P. intermedia*, SM, *S. mutans*, SA, *S. aureus*, SE, *S. epidermidis*, TD, *T. denticola*, EC, *E. corrodens*, TF, *T. forsythensis*; CP, *C. pneumoniae*.

Table 4
Mean levels of bacterial DNA in group B samples (one from each of nine elderly patients)

Species	AA	PG	PI	SM	SA	SE	TD	EC	TF	CP
Mean %	1.19	1.68	1.38	0.73	0.75	0.56	0.26	0.35	0.44	0.57
% positive samples	55.5	88.8	77.7	44.4	55.5	33.3	33.3	22.2	22.2	37.5

Abbreviations are as in Table 3.

sample size variability. Also, since the bacterial DNA appears to be only a fraction of the total DNA available, there is no obvious interference using the method.

In a recent communication, no periodontal bacterial DNA was detected in 52 specimens using standard PCR [35]. Our data confirm the applicability of Q-PCR as a preferred method for analysis of microbial component of total human DNA. We also detected a higher overall prevalence of most periodontal organisms than described by Haraszthy et al. [23], and most recently by Ishihara et al. [28]. This again may be due to our more sensitive method of analysis. The use of real-time PCR assay has previously proved to be particularly suitable for the specific and quantitative detection of a low DNA copy number in conventional PCR-negative samples [36].

The lower level of detection in the younger individuals (Group A) is probably due to the relatively young age of the source of these post mortem specimens compared to most studies where endarterectomy tissues are analyzed. In this respect, our study specifically addresses the issue of age. Periodontal diseases are a significant public health burden in the United States. Severe periodontal diseases (having 4 mm or more of attachment loss) increase in prevalence with age, with approximately 50% of 55–64 years old having evidence of severe disease. By age 75, roughly two-thirds of all adults have severe periodontitis and one-third of them have 6 mm or more of attachment loss (http://grants1.nih.gov/grants/guide/rfa-files/RFA-DE-04-001.html). Coincidentally, this is the age group with the highest incidence of infarction and stroke. Our data demonstrate that the elderly individuals (group B, mean age 67 years) have higher incidence of periodontopathogens in their plaques than the younger individuals (group A), as seen in Table 4 vs. Table 3 as well as in Fig. 1.

Species from the *Bacteroides* family were found in about 17% of the young but in about 80% of the elderly patients, as expected given the association of this family with adult and refractory periodontitis. Contrastingly, we found that *A.a.* is predominant in the young individuals (group A), also as expected, given its association with localized juvenile periodontitis. Additionally, it is notable that the incidences for the non-oral organisms, *S. epidermadis* and *S. aureus*, are among the lowest (see Tables 3–5). Notably, the mean number of bacteria per specimen is highest for *P. gingivalis*, although not by a big margin as shown in Table 5. These numbers provide evidence that the bacterial DNA found in atheromas is not due to a contamination from bacteremic blood in the vascular channels or hemorrhagic material. The

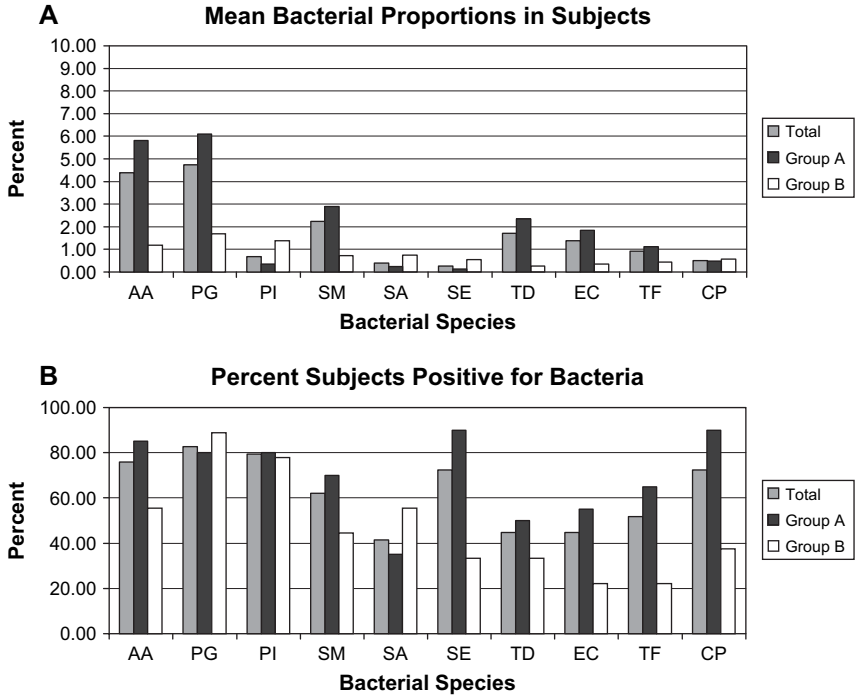

Fig. 1. Bar graphs summarizing the proportion of each species as proportion of total bacterial DNA (A) and the prevalence of the tested organisms in the two groups (B). Abbreviations are as in Table 3.

Table 5
Mean number of bacterial genomes

	AA	PG	PI	SM	SA	SE	TD	EC	TF	CP
Mean	2.03×10^6	2.19×10^6	3.15×10^5	1.03×10^6	1.87×10^5	1.23×10^5	7.90×10^5	6.41×10^5	4.24×10^5	2.32×10^5

Abbreviations are as in Table 3.

numbers are in line with recent findings demonstrating the presence of polymicrobial consortia in buccal epithelial cells [37]. In addition, the data confirm our own observations of live invasive periodontal bacteria in atheromatous plaque [29]. Notably, the bacteria that were visualized in that study, *P. gingivalis* and *A.a.*, were among the most prevalent periodontal bacteria in plaques.

This is not the only study to find DNA sequences of oral organisms in a majority of cardiovascular specimens studied [38]. However, methodology alone may not account for the varying results in studies to date [38–40]. Due to the circumstances surrounding specimen collection, studies to date have included relatively small numbers of total specimens, although our study does include sufficient numbers for statistical analysis. Additionally, the specimens collected in our study and the published literature represent populations from diverse geographic locations [38–40]. This in and of itself is enough to account for variability in oral bacterial colonization [41]. Primers used to detect and identify the organisms vary in the literature as well. While the exact specificity of primers can never be completely determined, the primer sequences we used in this study were tested for specificity by DNA data base analysis and subsequent testing with 40–50 strains of bacteria, including the species tested in this study, without evidence of cross-reactivity. We are equally confident of the primer sequences obtained from the literature (see references in Table 2). In short, the experimental identification of oral pathogens in cardiovascular (atheroma) specimens is still in its infancy. However, refinements in techniques and analysis lead to promising results to advance the field.

This is the first report to analyze for the presence of *C. pneumoniae* in parallel with periodontal and commensal pathogens. As seen in the data, *C. pneumoniae* prevalence is close in both age groups. Most importantly, the level of *C. pneumoniae* DNA is low in comparison with that of the periodontal pathogens (Tables 3–5), even using this very sensitive analysis. This is in agreement with the controversial role of *C. pneumoniae* in vascular inflammations [42] and supports the primary importance of the periodontal infections as a contributing factor in vascular inflammations. It is well accepted that inflammation is a hallmark of periodontal disease. However, while we are using the presence of inflammation as a hallmark of disease in this study, we are not attempting to measure the level of inflammation.

This is also the first report to analyze for the presence of as many as ten bacterial pathogens in atheromatous plaques. We conclude that (1) there continues to be evidence that oral bacteria can be frequently found in atherosclerotic lesions, (2) the prevalence and the proportion are higher in elderly patients, (3) the number of bacteria demonstrates their association with the tissue and not with the vascular blood, (4) *C. pneumoniae* is a minor component of the bacterial population and (5) quantitative PCR is an appropriate method for the quantitative detection of bacterial species in these tissues.

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