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Periodontal pathogens in atheromatous plaques isolated from patients with chronic periodontitis

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Background and Objective: It has been suggested that chronic infections may predispose to cardiovascular disease. The relationship between periodontal disease and cardiovascular disease has been a subject of increasing research in recent years. The isolation and identification of periodontal bacteria from atheromatous plaque can contribute to our knowledge of this vascular disease. The aim of this study was to isolate and identify periodontal bactera from the periodontal pockets of different patients and to compare them with the microorganisms detected in the atheromatous plaques obtained from the same patients.

Material and Methods: Clinical isolates were obtained from 12 patients with periodontal wounds and atheromathous plaques. These samples were cultured in the appropriate bacteriological culture media and incubated in an anaerobic system. Periodontal bacteria were identified using polymerase chain reaction (PCR) assays.

Results: From the 12 patients studied, nine presented different periodontopathic bacterial species. In two, *Actinobacillus actinomycetemcomitans* was present in the periodontal pockets and the respective atheromatous plaques.

Conclusion: The presence of *A. actinomycetemcomitans* in atheromatous plaques and the periodontal pockets of the same patients could indicate a role for periodontal pathogenic bacteria in the atherosclerosis disease process.

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Periodontitis is a common disease that affects individuals of different ages and is characterized by a chronic tissue-destructive inflammation that diminishes the attachment apparatus of the teeth, inducing tooth loss and, eventually, edentulousness (1). The pathogenesis of periodontal disease is thought to derive from the accumulation of subgingival bacterial species, predominantly gram-negative, anaerobic and microaerophilic bacteria (2). The global prevalence of periodontal diseases is high, and severe forms of chronic periodontitis affect ≈10–15% of individuals in most populations (1).

The relationship between periodontal diseases and systemic diseases has been documented previously (3). Evidence from recent epidemiologic studies suggests a link between periodontal infections and an increased risk of atherosclerosis and related cardiovascular and cerebrovascular events in humans (4,5). There are several hypotheses that explain the association between periodontal disease and complications of atherosclerosis (6,7), but there is still no concluding scientific evidence on this relationship. In this context, it is important to consider that cardiovascular disease is the main cause of morbidity and mortality

in the Western civilization (8). This disease may be on the increase as a result of changes in the lifestyle of the population, a factor that reduces life expectancy. Thus, efforts to help control atherosclerosis are an important public health issue. The principal aim of this study was to detect the presence of periodontopathic bacterial species in the periodontal pockets of patients with periodontal disease and to relate these findings with periodontal microorganisms detected in atheromatous plaques obtained from the same patients.

Materials and methods

Patients and clinical samples

Twelve patients with chronic periodontitis and a clinical diagnosis of obliteration of inferior extremities or carotid artery stenosis were selected. The age of patients ranged between 56 and 73 years. All patients were hospitalized in the Service of Vascular Surgery in the Regional Hospital of Talca (Chile). Six patients presented clinical, radiological and laboratory studies that correspond to carotid artery stenosis: two presented popliteal artery, two tibia artery, and two femoral artery obliteration. The atheromatous plaques were obtained by means of a surgical endarterectomy process and were performed 1 wk after the periodontal examination. The atheromatous samples were placed in a sterile tube with 10 ml of saline solution and immediately transferred to the Laboratory of Microbiological Research, University of Talca. The odontological examinations were performed by a periodontist who determined the periodontal pocket depth, plaque index (9), clinical attachment level and bleeding on probing in six sites per tooth. All patients showed radiographic evidence of alveolar loss. A sample of the pocket of each tooth was collected using a sterile paper tip point. This tip was inserted into the pocket for 10 s, then transferred to a sterile tube and sent to the Laboratory of Microbiological Research, University of Talca. Informed consent was obtained from all the patients studied before the investigation. None received any antibiotic treatment 6 months before the examination.

Bacteriological study

All microbiological procedures were performed in a safety cabinet (Nuaire Class II; Plymouth, MN, USA). With the aim to obtain a wide range of anaerobic bacterial growth, two bacteriological culture media were used. All paper tip points were plated onto Brucella blood agar (Difco, Detroit, MI, USA) supplemented with defibrinated sheep blood (5%), vitamin K (1 μg/ml), hemin (5 μg/ml) and kanamycin (50 μg/ml), and onto Brucella blood agar (Difco) supplemented with defibrinated sheep blood (5%), vitamin K (1 μg/ml) and vancomycin (7.5 μg/ml). *Actinobacillus actinomycetemcomitans* was cultured in Brucella agar supplemented with vitamin K and vancomycin. The seeded Petri dishes were placed in an anaerobic system (bio-Merieux, Lyon, France) and incubated at 37°C for 72 h. The atheromatous plaques were homogenized carefully in the same transported sterile tube and 100 μl of this sample was plated onto several plates containing the same bacteriological culture media described above, and cultured under the same physicochemical conditions described above. The sampling was repeated three times for each atheromatous plaque.

Bacterial identification by polymerase chain reaction

All colonies that grew on the bacteriological cultures were identified by means of polymerase chain reaction (PCR). Genomic DNA was extracted from one colony, as described in Aqua Pure Genomic DNA Isolation kit (Bio-Rad, Hercules, CA, USA) and the DNA concentration was determined spectrophotometrically. The primers utilized were designed in accordance with Ashimoto *et al.* (10) and were synthesized by Omega Bio-tek (Victoria, BC, Canada). PCR amplification was carried out in a 25-μl reaction mix containing 10 mM Tris HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 0.5 U *Taq* DNA polymerase, 0.4 μM of each primer pair and 15 ng of DNA template. Amplification was performed according to Avila & Velasquez (11), in a DNA thermal cycler (Thermo Hybaid, Ashford, UK) programmed to 94°C (5 min), followed by 30 cycles at 94°C for 30 s. Annealing temperature for each primer pair (see Table 1) for 30 s, then 72°C for 30 s, then 72°C for 5 min to allow the completion of DNA extension.

A negative control reaction without template DNA was included in each PCR run. The strains *A. actinomycetemcomitans* ATCC 29523, *Porphyromonas gingivalis* ATCC 33277, *Tannarella forsythia* ATCC 43037 and *Prevotella intermedia* ATCC 25611 were used as control for genomic DNA. The PCR products were analysed by electrophoresis in 1% agarose gel, stained with ethidium bromide(0.5 μg/ml) and photographed under ultraviolet (UV) light.

Table 1. Primers used to identify the bacteria

Bacteria	Oligonucleotide sequence 5′ → 3′	PCR annealing temperature (°C)	Amplicon length (kb)
Actinobacillus actinomycetemcomitans	GCTAATACCGCGTAGAGTCGT ATTTCACACCTCACTTAAAGGT	50	0.5
Tannerella forsythia	GCGTATGTAACCTGCCCGCA TGCTTCAGTGTCAGTTATACCT	60	0.6
Porphyromonas gingivalis	AGGCAGCTTGCCATACTGCG ACTGTTAGCAACTACCGATGT	60	0.4
Prevotella intermedia	TTTGTTGGGGAGTAAAGCGGG TCAACATCTCTGTATCCTGCGT	50	0.6

PCR, polymerase chain reaction.

Table 2. Periodontal pocket depth, plaque index and bleeding on probing in patients with chronic periodontitis and clinic diagnostic of carotid artery stenosis or artery obliteration of inferior extremities

Patients			Diseases					
No.	Gender	Age	Carotid stenosis	Obliteration of inferior extremities	Periodontitis	Pocket depth (mm)	Plaque index	Bleeding on probing %
1	M	61	+		+	5.2	2.2	34
2	M	70		+	+	4.1	1.6	26
3	F	56	+		+	4.8	2.1	30
4	M	64		+	+	4.0	1.8	28
5	F	59	+		+	5.9	2.5	35
6	F	73	+		+	4.2	1.9	29
7	F	69		+	+	3.9	1.5	27
8	F	65		+	+	4.7	2.0	31
9	M	62	+		+	4.1	2.0	30
10	F	63		+	+	5.0	2.2	33
11	M	66		+	+	4.9	2.1	30
12	M	70	+		+	5.8	2.4	35

+, presence of disease; F, female; M, male.

Results

All 12 patients presented with chronic periodontitis, with pocket depths of 3.9–5.9 mm. The plaque index ranged from 1.5 to 2.5, and the bleeding on probing was 27–35% (Table 2). Bacterial growth with different morphological colonies was observed in all of the periodontal samples studied. However, colony growth was observed in two samples of atheromatous plaques. All colonies with different morphologic characteristics were investigated by PCR (Fig. 1). Some colonies were impossible to identify, by PCR, in periodontal samples from three patients (Table 3). In nine samples, an association between two or three periodontal bacteria was observed and *P. gingivalis* was the most common (Table 3). *T. forsythia* was only isolated in two periodontopathic cases. One sample of atheromatous plaques obtained from carotid artery stenosis, and the other from a tibia artery case, showed growth of *A. actinomycetemcomitans*. These patients also presented the same bacterial species in their periodontal samples, among other microorganisms (Table 3). In general, the bacteriological media utilized was a good substrate for culturing anaerobic bacteria, but it was observed that *A. actinomycetemcomitans* only grew in Brucella agar supplemented with vitamin K and vancomycin. The other bacterial species grew only in the Brucella agar supplemented with vitamin K, hemin and kanamycin. The colonies that could not be identified by PCR were grown in this same bacterial culture media.

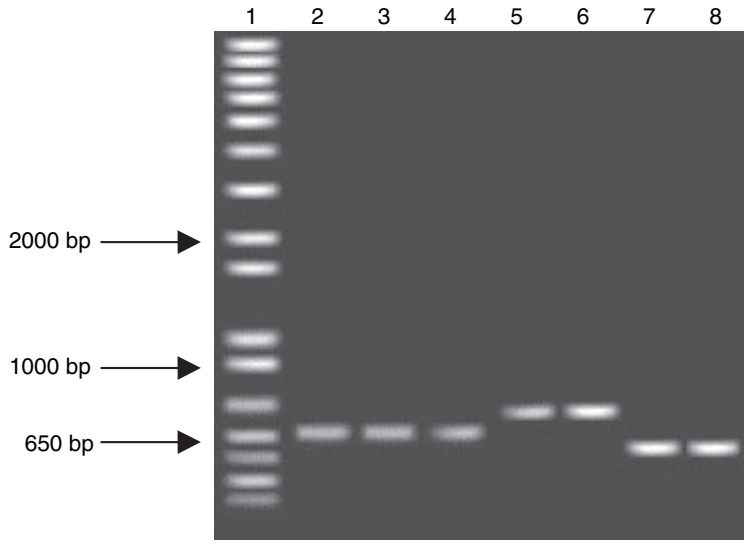

Fig. 1. Identification of bacterial species from periodontal and atheromatous plaques by polymerase chain reaction (PCR). Lane 1, 1-kb Plus DNA ladder (molecular weight marker); PCR products: lanes 2 and 3, *Actinobacillus actinomycetemcomitans* (clinical isolates); lane 4, *A. actinomycetemcomitans* ATCC 29523; lane 5, *Prevotella intermedia* (clinical isolate); lane 6, *P. intermedia* ATCC 25611; lane 7, *Porphyromonas gingivalis* (clinical isolate), and lane 8, *P. gingivalis* ATCC 33277.

Discussion

Several microorganisms, including *Chlamydophila pneumoniae* and cytomegalovirus, have been implicated in the infectious aetiology of atherosclerosis (12). Several investigations have attempted to relate periodontitis to cardiovascular diseases (13,14). In this work, we selected a group of patients with two types of pathologies: a chronic periodontitis in addition to obliteration of inferior extremities or carotid artery stenosis. It was possible to isolate bacteriological colonies from 12 periodontal samples, but bacteria were identified in only nine. Therefore, PCR was found to be a better method for using to identify *A. actinomycetemcomitans* than presumptive biochemical identification and should be the preferred method for the detection of subgingival plaque microorganisms (15). In three cases, colonies could not be identified, probably because adequate primers for PCR were not available. It is interesting to point out that several colonies of *A. actinomycetemcomitans* were isolated in an atheromatous plaque from carotid artery stenosis and in another from obliteration of inferior extremities. This microorganism has been detected by PCR in atheromatous plaque in previous investigations but it has not been cultured (12). The bacteriological culture and the PCR identification of this microorganism offer scientific evidence

Table 3. Bacterial species present in the periodontal and atheromatous plaque samples

Patient no.	Periodontal samples	Atheromatous plaque samples
1	*P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*	
2	*P. intermedia*, *A. actinomycetemcomitans*	
3	*T. forsythia*, *P. gingivalis*, *A. actinomycetemcomitans*	*A. actinomycetemcomitans*
4	*P. intermedia*, *A. actinomycetemcomitans*	
5	*A. actinomycetemcomitans*, *P. gingivalis*	
6	–	
7	*T. forsythia*, *P. gingivalis*	
8	*A. actinomycetemcomitans*, *P. gingivalis*	*A. actinomycetemcomitans*
9	–	
10	*P. intermedia*, *P. gingivalis*, *A. actinomycetemcomitans*	
11	*P. gingivalis*, *P. intermedia*	
12	–	

A. actinomycetemcomitans, *Actinobacillus actinomycetemcomitans*; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *T. forsythia*, *Tannerella forsythia*.

of its possible role in the atherogenesis process. *A. actinomycetemcomitans* has been suggested to contribute to the pathogenesis of coronary heart disease, and antibody levels against this pathogen in serum have been viewed as indicators of the risk for this disease (16). It is difficult to determine the reasons for the absence of *P. intermedia* and *P. gingivalis* growth in atheroma samples, although these bacteria are usually present in periodontal samples.

Chronic periodontal infection and its association with myocardial infection have been studied in great detail in the last few years, but complete evidence has not yet been established (17). However, it is possible to argue that periodontal bacteria, particularly gram-negative bacteria, can increase the risk of cardiac damage. *A. actinomycetemcomitans*, a gram-negative coccobacillus, is an important etiological agent of different forms of periodontitis (18). Chronic periodontitis is characterized by multiple episodes of bacteraemia, events that can allow *A. actinoycetemcomitans* to migrate to the atheromatous plaque. As a result of the small number of patients included in this work, it is not possible to obtain conclusive statistical results; nevertheless, the presence of *A. actinomycetemcomitans* in the periodontal samples and in the atheromatous plaques of the same patients, support the potential role of this periodontopathogenic bacterial species in some step of the atherogenesis process or as a contributor of a different mechanism that worsens this disease. However, if this association is casual, a deeper evaluation of periodontal diseases is necessary. This work opens new insights regarding the potential risk of *A. actinomycetemcomitans* for atherosclerosis. Further molecular studies are required for a better understanding of this association.

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References

1. Buhlin K, Gustafsson A, Graham A, Frostegärd J, Klinge B. Risk factors for cardiovascular disease in patients with periodontitis. *Eur Heart J* 2003;**24:**2099–2107.
2. López N, Smith P, Gutierrez J. Higher risk of preterm birth and low birth weigth in women with periodontal disease. *J Dent Res* 2002;**81:**58–63.
3. Haynes W, Stanford C. Periodontal disease and atherosclerosis. From dental to arterial plaque. *Arterioscler Thromb Vasc Biol* 2003;**23:**1309–1311.
4. Lalla E, Lamster IB, Hofman MA *et al.* Oral infection with a periodontal pathogen accelerates early atherosclerosis in apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol* 2003;**23:**1405–1411.
5. Cueto A, Mesa F, Bravo M, Ocaña-Riola R. Periodontitis as risk factor for acute myocardial infarction. A case control study of Spanish adults. *J Periodont Res* 2005;**40:**36–42.
6. Wu T, Trevisan M, Genco R, Falkner KL, Dorn JP, Sempos CT. Examination of the relation between periodontal health status and cardiovascular risk factors: serum total and high density lipoprotein cholesterol, C-reactive protein, and plasma fibrinogen. *Am J Epidemiol* 2000;**71:**1528–1534.
7. Slade G, Offenbache S, Beck J, Heiss G, Pankow J. Acute phase inflammatory response to periodontal disease in the US population. *J Dent Res* 2000;**79:**49–57.
8. Lowe G. The relationship between infection, inflammation and cardiovascular disease: an overview. *Ann Periodontol* 2001;**6:**1–6.
9. Daly C, Mitchell D, Highfield J, Grossberg D, Stewart D. Bacteremia due to periodontal probing: a clinical and microbiological investigation. *J Periodontol* 2001;**72:**210–214.
10. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;**11:**266–273.
11. Avila-Campos M, Velasquez-Melendez G. Prevalence of putative periodontopathogens from periodontal patients and healthy subjects in Sao Paulo, SP, Brazil. *Rev Inst Med Trop Sao Paulo* 2002;**44:**1–5.
12. Haraszthy V, Zambon J, Trevisan M, Zeid M, Genco R. Identification of periodontal pathogens in atheromatous plaques. *J Periodontol* 2000;**71:**1554–1560.
13. Mattila K. Does periodontitis cause heart disease? *Eur Heart J* 2003;**24:**2079–2080.
14. Cairo F, Gaeta C, Dorigo W *et al.* Periodontal pathogens in atheromatous plaques. A controlled clinical and laboratory trial. *J Periodont Res* 2004;**39:**442–446.
15. Flemming T, Rüdiger S, Hofman U *et al.* Identification of *Actinobacillus actinomycetemcomitans* in subgingival plaque by PCR. *J Clin Microbiol* 1995;**33:**3102–3105.
16. Pussinen P, Nyyssönen K, Alfthan G, Salonen R, Laukkanen JA, Salonen JY. Serum antibody levels to *Actinobacillus actinomycetemcomitans* predict the risk for coronary heart disease. *Arterioscler Thromb Vasc Biol* 2005;**25:**833–838.
17. Hujoel P, Drangsholt, M, Spiekerman C, DeRouen T. Pre-existing cardiovascular disease and periodontitis: a follow-up study. *J Dent Res* 2002;**81:**186–191.
18. Suzuki N, Nakano Y, Yoshida Y, Ikeda D, Koga T. Identification of *Actinobacillus actinomycetemcomitans* serotypes by multiplex PCR. *J Clin Microbiol* 2001;**39:**2002–2005.