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RESEARCH ARTICLE

Quantitative Detection of Periodontopathogenic Bacteria in Atherosclerotic Plaques from Coronary Arteries by Real-Time PCR

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Abstract:

Objectives:

Epidemiologic studies have suggested periodontitis as a risk factor for Coronary Artery Diseas (CAD). Detection of periopathogens in atheromatous plaque provides some evidence for the causal relationship between these two conditions. The aim of this study was to determine the presence and quantity of periopathogens in coronary atherosclerotic plaques in patients undergoing Coronary Artery Bypass Graft (CABG) surgery.

Methods:

20 patients who were candidates for endarterectomy were enrolled in this study for the periodontal examination. Subgingival and coronary atherosclerotic plaque samples were then collected. Thereafter, quantitative detection of *Aggregatibacter actinomycetemcomitans (A.a), Porphyromonas gingivali (P.g)*, and all bacteria detected by Real-Time PCR (RT-PCR) were measured. The correlation analysis was also used to evaluate the relationship between quantities of periopathogens in atherosclerotic and subgingival plaque samples.

Results:

A.a was detected in 13 patients (65%) with subgingival plaques and 4 patients (20%) with atherosclerotic plaques. In addition, *P.g* was found in 15 patients (75%) with subgingival and 10 patients (50%) with atherosclerotic plaques. *A.a* represented means of 2.7% and 10.04% of detected bacteria in both atherosclerotic and subgingival plaque samples, respectively. The mean of quantity of *P.g* was 10.85% and 12.87% of the detected bacteria obtained from atherosclerotic and subginigival samples, respectively. Correlation analysis showed a significant correlation between the quantities of *A.a* in the atherosclerotic and subgingival plaques, but such a significant relationship was not found for *P.g*.

Conclusion:

This study confirmed the detection of *A.a* and *P.g* in atheromatous plaque. The quantitative data suggested that periopathogens comprise a significant proportion of atherosclerotic plaque microbiome, which may consequently contribute to the development of CAD.

Keywords: Atherosclerotic plaque, Periodontal pathogens, Subgingival plaque, Real-Time PCR, Coronary Artery Diseas (CAD), Coronary Artery Bypass Graft (CABG).

1. INTRODUCTION

Cardiovascular Disease (CVD) is one of the most prevalent causes of death worldwide. A cohort study performed in 2013 reported that CVD caused more than three million deaths among the population younger than the age of 60 and isresponsible for about 30% of all deaths worldwide [1].

Previous studies showed that a higher prevalence of the atherosclerotic disease is among patients with inflammatory diseases such as periodontitis. Several studies have also shown

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that periodontitis is independently associated with athero-sclerosis [2]. Periodontitis is a chronic inflammation of periodontium and its advanced form is characterized by alveolar bone loss, which could consequently lead to tooth loss [3]. According to the epidemiologic data, the periodontal disease is affecting 63-68% of the population and in patients aged 40 years, a mean of 76% of teeth is extracted due to periodontal disease [4].

The result of previous studies showed that periodontitis might be associated with the atherosclerotic disease even after the adjustment for known risk factors such as smoking and diabetes [5]. Some authors proposed that except for the common risk factors such as diabetes, smoking, socio-economic factors, and high blood pressure, inflammation like chronic periodontitis is suggested as another factor contributing to the development of CVD [6].

Although the causal mechanism by which periodontitis plays a role in the formation and development of coronary atherosclerotic plaque, has not been fully elucidated yet, inflammatory mediators, bacterial invasion, and the altered lipid metabolism were proposed as the plausible biological links between these two diseases [7].

Previously, the effect of direct infection of the endothelial layer of vessels by *Chlamydia pneumonia* and *Cytomega-lovirus* on atherogenesis has been widely accepted [8]. However, it is not completely clear yet, whether the periopathogens directly contribute to atheromatous plaque formation. The presence of oral pathogens at the diseased site is one of the perquisites to suggest this role. Bacteremia via the ulcerated periodontal pockets may provide a possible pathway for colonization of periopathogens in atherosclerotic plaque [7].

In order to detect the role of periopathogens in atheromatous plaque, many investigations have been performed. Although periodontal pathogens have been detected in atherosclerotic plaque samples using various methods, a correlation between the quantitative amount of periopathogens in coronary atherosclerotic plaque and periodontal pockets has not been cleared yet.

The present study was performed to detect and assess the quantity of two putative periodontal pathogens including *Porphyromonas gingival (P.g)* and *Aggregatibacter actinomycetemcomitans (A.a)* in the samples obtained from subgingival and coronary atherosclerotic plaques, which were harvested from chronic periodontitis patients who were selected as candidates for endarterectomy or CABG surgery by RT-PCR.

2. MATERIALS AND METHODS

2.1. Study Design

This study was a cross-sectional study, which included those subjects presenting periodontal disease concomitantly with obstructive coronary artery atherosclerosis (OCAA) who were candidates for endarterectomy or CABG surgery at Imam Reza Hospital (Mashhad, Iran), between November 2017 and November 2018. This study was approved by the Ethics Committee of Mashhad Dental School, the State University of Mashhad (under number IR.mums.sd.REC.139057), and all the included subjects signed a consent form prior to the study.

2.2. Periodontal Examination

One day before cardiac surgery, all the patients underwent periodontal examination by a single trained periodontist who collected periodontal data, including Probing Depth (PD), Attachment Loss (AL), and Bleeding on Probing (BOP). The subjects aged between 40 and 80 years old with at least 10 remaining teeth and AL ≥ 3 mm and PD ≥5 mm seen in at least 30% of all sites were considered as periodontitis patients and then entered the study. Moreover, those subjects with a history of periodontal treatment or who underwent antibiotic therapy during the three months prior to this study were excluded.

2.3. Subgingival Plaque Collection

One day before CABG surgery, subgingival plaque samples were taken from the deepest periodontal pocket with PD ≥ 5 mm for microbial analysis. Before sampling, the teeth were dried with sterile gauze and supragingival plaque was then removed. Afterward, the subgingival plaque samples were obtained with Sterile curettes placed in micro-centrifuge tubes containing EDTA solution that were stored at -70°C.

For DNA extraction, the samples were suspended in 500 μl of lysis buffer (100 mM Tris HCL, 0.05% Tween 20, 0.24 mg/ml Proteinase K, pH 8.5), incubated for 1 h at 60°C, heated for 15 min at 97°C to inactivate the Proteinase K, boiled for 10 min, and finally centrifuged to remove cell debris. The supernatant was used as a DNA template for RT-PCR analysis.

2.4. Atherosclerotic Plaque Collection

During the surgery of CABG, the surgeon excised a bit of atherosclerotic plaque from the obstructed coronary artery with the least size of 0.5-1 mm. Thereafter, the plaque samples were placed in microcentrifuge tubes containing EDTA solution and then stored at -70°C until analysis. Approximately, 100 mg of tissue was homogenized and then subjected to DNA extraction. The DNA was extracted from these tissue samples using the tissue DNA extraction kit (Parstous, Mashhad, Iran) [8] according to the manufacturer's instructions. Finally, the extracted DNA was measured by spectroscopy.

2.5. Quantitative Analysis by RT-PCR

The primer/probe sets for periodontal bacteria were selected (Maeda *et al.* (2003) [9] and Nonnenmacher *et al.*, (2004) [10]), and used in this study as described in Table **1**.

Table 1. Primers and probes used in the RT-PCR.

	Primer or Probe Sequence (5' to 3')	Primer or Probe Name
Maeda *et al.* (2003)	CTTACCTACTCTTGACATCCGAA	A.A Forward
	ATGCAGCACCTGTCTCAAAGC	A.A Reverse
	CY5AGAACTCAGAGATGGGTTTGTGCCTTAG'BHQ2	A.A Probe
Nonnenmacher *et al.* (2004)	TGCAACTTGCCTTACAGAGGG	P.G Forward
	ACTCGTATCGCCCGTTATTC	P.G Reverse
	JOE AGCTGTAAGATAGGCATGCGTCCCATTAGCTA BHQ1	P.G Probe
Nonnenmacher *et al.* (2004)	TGGAGCATGTGGTTTAATTCGA	Universal Forward
	TGCGGGACTTAACCCAACA	Universal Reverse
	FAM CACGAGCTGACGACA(AG)CCATGCA BHQ1	Universal Probe

Table 2. Volume of reagents used in the RT-PCR.

Volume	Reagents
12.5 µl	PCR Mix 2X
0.8 µl	Aa Forward Primer (10 µM)
0.8 µl	Aa Reverse Primer (10 µM)
1 µl	Aa Probe
1 µl	Pg Forward Primer (10 µM)
1 µl	Pg Reverse Primer (10 µM)
1 µl	Pg Probe
1 µl	Universal Forward Primer (10 µM)
1 µl	Universal Reverse Primer (10 µM)
1 µl	Universal Probe
1.9 µl	H2O
2 µl	DNA
25 µl	total volume

RT-PCR assays were performed in a total volume of 25 µl containing 12.5 µl 2X Taqman Universal Master Mix (Applied Biosystems), 0.8 µl each forward and reverse primer (final concentration 10 µM each), 1 µl Taqman probe (final concentration 100 nM), 2 µl template DNA solution, and 1.9 µl of sterilized DNase- and RNasefree water (Table **2**) using a Rotor-Gene 6000. For the detection of bacterial DNA for *A.a* and *P.g,* an initial denaturation for 10 min at 95°C was followed by 40 cycles for 15 s at 95°C and for 1 min at 60°C. The negative control was all PCR ingredients with no DNA.

2.6. Statistical Analysis

Mean values ± SD were calculated for each bacterial species and also for detected eubacteria. Software SPSS, version 13, was used for statistical analyses. Kolmogorov Smirnov test was used to determine if data were distributed normally. The relationship between the number of periopathogens in atherosclerotic and subgingival plaques was evaluated using Pearson and Spearman correlation tests. The level of significance was set as P value ≤0.05.

3. RESULTS

The study group consists of 20 patients, 17 men (85%) and 3 women (15%), with a mean age of 57.25±8.28 years old (ranged from 43 to 72 years old). Notably, 8 patients (40%) were smokers. The patients exhibited an average of 17.0±8.18 teeth. The mean probing depth was 4.96±0.78 and more than 52% of the patients exhibited ≥5 mm probing depth.

In this study, of 20 patients, we detected *A.a* and *P.g* in 13 patients (65%) and 15 patients (75%), took subgingival plaque samples and atherosclerotic plaque samples from 4 patients (20%) and 10 patients (50%), respectively. The concomitant detection of *A.a* and *P.g* was observed in 18 subgingival plaque samples (90%) and 10 atherosclerotic plaque samples (50%). In one subgingival plaque sample and 10 atherosclerotic plaque samples, we detected none of these two bacteria (Table **3**).

Table 3. Quantities of A.a and P.g in Atheromatous and Subgingival Plaques from 20 patients who were candidate for endarterectomy or CABG surgery.

Microorganism	Atheromatous Plaque (%)	Subginbival Plaque (%)
A.a		
Positive	**4(20%)**	**13(65%)**

(Table 3) contd.....

Negative	16(80%)	6(35%)
P.g		
Positive	10(50%)	15(75%)
Negative	10(50%)	4(25%)
P.g and A.a		
Positive	10(50%)	18(90%)
Negative	10(50%)	1(5%)

Table 4. Mean percentage of *A.a* and *P.g* from bacteria detected in subgingival and atherosclerotic plaque samples and the correlation between the quantity of *A.a* and *P.g* in subgingival and atherosclerotic plaques.

Percentage Distribution of Bacteria	Atherosclerotic Plaque	Subgingival Plaque	Coefficient Correlation (r, p-value)
A.a	**2.70±6.05**	**10.04±8.78**	**p-value:0.01, r:0.6**
P.g	**10.85±13.90**	**12.78±12.22**	**p-value:0.07, r:0.42**
A.a and P.g	**13.55±18.75**	**22.89±15.57**	**p-value:0.02, r:0.53**

In our study, the quantitative data of RT-PCR showed that *A.a* comprised a mean of 2.7% of the bacteria represented in atheromas. In addition, *P.g* comprised a mean of 10.85% of the microbiome of the atherosclerotic samples. According to the result of the Kolmogorov-Smirnov (KS) test, all variables used for correlation analysis were normally distributed, except *A.a* quantity in atherosclerotic plaques and the bacteria detected in subgingival plaques. A statistically significant correlation was also found between the quantity of *A.a* in subgingival and atherosclerotic plaques using a Spearman's correlation test (p-value: 0.01, r: 0.6). Due to the result of the Pearson test, such a significant relation was not found for *P.g* (p-value:0.07). According to the result of the Pearson test, total quantities of *A.a* and *P.g* in subgingival and atherosclerotic plaques were significantly correlated (p-value: 0.02, r: 0.53); however, the result of Spearman's test showed that the relationship between the quantities of bacteria detected by RT-PCR in subgingival and atherosclerotic plaques was not significant (p-value: 0.09). By performing RT-PCR, the mean percentile quantities of *A.a* and *P.g* in both atherosclerotic and subgingival plaque samples and the results of correlation analysis are reported in Table **4**.

4. DISCUSSION

Periodontitis is a bacterial infection of supporting tissues of teeth, which is characterized by attachment and alveolar bone loss [3]. Severe chronic periodontitis is affecting more than 10% of the population, so it are considered a major health issue [11]. An association between periodontitis and a number of systemic conditions such as CVD, obesity, metabolic syndrome, hypertension, and adverse pregnancy outcomes has been observed by epidemiological studies. Accordingly, in many epidemiological studies, the association between the prevalence of periodontitis and CAD was significant even after the adjustment for similar risk factors such as smoking and diabetes [12]. In periodontitis, a sizeable ulcerated surface of periodontal pocket amounting up to 8-20 cm^2, is considered as a pathway for bacteria to enter the blood stream, which consequently predisposes the patient to the increased incidence of bacteremia [13].

Based on the previous experimental studies, periopathogens play a significant role both in the formation and development of coronary atherosclerotic plaques with direct (hijack dendritic cells, macrophages or erythrocytes invade endothelial cells by the aggregation of platelets) and indirect (up-regulating cell adhesion molecules, chemokines, and pro-inflammatory cytokine) mechanisms [14]. Controversial results have been reported about the colonization of periopathogens in coronary atheromatous plaques. Only a few studies assessed the number of periopathogens in subgingival plaques and atherosclerotic lesions, as well as the relationship between them [15 - 17]. In our study, the quantities of two periodontopathic bacteria, including *P.g* and *A.a* were measured in subgingival and coronary atherosclerotic plaques by RT-PCR. Correspondingly, *P.g* is considered as a key stone pathogen in the pathogenesis of periodontitis. Moreover, virulent factors and pathogenic ability of *P.g*, even at a low frequency, could induce the dysbiosis of microbial communities [18]. It is noteworthy that most of the in-vitro and animal studies used *P.g* as the model pathogen of periodontitis, and they have shown that *P.g* could invade the endothelial cells. The expression of pro-inflammatory cytokines, absorption of LDL, the formation of foam cell, and proliferation of smooth muscle cells in the infected intima layer accelerated the atherogenesis [19].

On the other side, *A.a* was found to be strongly associated with aggressive forms of periodontitis, so it could be associated with systemic infection [20]. *A.a* can invade nonphagocytic cells such as endothelial cells, and can also manipulate the host response by activating or inhibiting different signaling pathways [21]. According to the findings of Colhoun *et al.*, a higher level of IgG against *A.a* was associated with the increased risk of coronary atherosclerosis [22].

Most of the previous studies have used the PCR method for the detection of 16srRNA genes of periopathogens in atherosclerotic plaques. The detection of bacterial DNA in atherosclerotic plaque could be due to the attachment of microorganisms to the existing atherosclerotic lesion. PCR, fluorescence in situ hybridization (FISH), and DNA-DNA Checkboard hybridization can also detect both vital and non-vital microorganisms [23]. However, using the RT-PCR

method, it is possible to both quantify and detect microorganisms colonizing coronary atherosclerotic lesions. In this study, among the 20 patients, we detected *A.a* in 13 patients (65%) of subgingival plaque samples as well as in 4 patients (20%) of atherosclerotic plaque samples, and *P.g* in 15 patients (75%) of subgingival plaque samples as well as 10 patients (50%) of atherosclerotic plaque samples. Many studies used different methods to identify periopathogens in atherosclerotic plaque samples collected during endarterectomy. The results of these studies regarding the prevalence rates of *A.a* and *P.g* are summarized in Table **5**.

In our study, the quantitative data of RT-PCR showed that *A.a* comprised a mean of 2.7% of the microbial community observed in atherosclerotic plaque, and *P.g* represented 10.85% of bacteria detected in atheromas. According to the result of RT-PCR in Gaetti-jardim's study, 11.3% and 18.8% of total bacteria of the microbial community were *A.a* and *P.g* in atherosclerotic plaque, respectively [17]. In addition, *P.g* was found only in one atherosclerotic plaque sample obtained from a healthy periodontal subject, which comprised 7.2% of the detected bacteria.

The concomitant detection of *A.a* and *P.g* was observed in 2 samples of 39 periodontitis patients, which represented a mean of 23% of detected bacteria. In this study, only 4 samples showed the concomitant presence of *A.a* and *P.g*, which comprised 41% of all bacteria detected in atherosclerotic plaque. Notably, PCR Method can detect a certain microorganism in the atherosclerotic lesion, but this does not exactly mean that bacteria might play a specific role in the formation and development of atherosclerotic plaques. The evaluation of the amount of certain bacteria by RT-PCR in the atherosclerotic plaques was considered as a better estimation of the effect of microorganisms on the pathogenesis of atherosclerotic diseases, compared to PCR. According to the result of correlation analysis, the amount of *A.a* in subgingival plaque was significantly related to the amount of bacteria in atherosclerotic plaque, but such a significant relationship was not found for *P.g*. Moreover, the detection of *A.a* in atherosclerotic plaque may be dependent on the frequent bacteremia and *A.a* could attach to the already existed atherosclerotic lesion. Correspondingly, this means that the decreased bacterial load in the primary colonization site

(subgingival plaque) after periodontal treatment could reduce the quantity of *A.a* in the secondary colonization site (atherosclerotic plaque). On the basis of this study results, in all subjects, *A.a* was detected in both of the atherosclerotic and subgingival plaque samples simultaneously.

The quantity of *P.g* in atherosclerotic samples was not significantly related to the subgingival plaque. In 10% of the subjects with *P.g* positive atheroma plaque, *P.g* was not found in their subgingival samples. These findings proposed that the colonization of *P.g* in atherosclerotic plaque was independent of the bacterial quantity in subgingival plaque. In addition, some unknown factors like bacterial virulence might play a role in the colonization of *P.g* in distant sites. According to the result of the study by Figuero *et al.*, the frequency of the detection of *A.a* DNA was significantly more in atherosclerotic samples of dentate subjects compared to edentulous patients. However, this relationship was not significant for *P.g* [24]. The atheromatous plaque formation and periodontitis are chronic processes, and changes in the microbial population may be expected during this time. It is still unclear how changes in the quantity of microorganisms in subgingival plaque affect the atherosclerotic plaque microbiome. Our results suggest that after the initial colonization in a distant site, *P.g* could independently survive. *P.g* has several virulent factors, so it can confront the host defense mechanisms. Additionally, it has the capacity to change the microbial ecologic condition as well as a competitive advantage in comparison to the other microorganisms of the bacterial niche. Considering all these characteristics, *P.g* was recognized as the keystone pathogen in the pathogenesis of periodontitis and it might also play the same role in the microbiome of atherosclerotic plaque. Factors affecting the number of periodontopathic bacteria after the initial colonization in atherosclerotic plaque have not been investigated yet. It seems difficult to answer the question of whether the detection of periopathogens is the result of attachment to the already existed atherosclerotic lesion or these bacteria play an active role in the formation and development of atherosclerotic plaque.

Answering these questions requires performing further experimental animal studies. It seems that Metagenomic studies may provide a better understanding of microbial community and ecological functions of periopathogens in atherosclerotic plaque [25 - 30].

Table 5. Percentile prevalence DNA detection of *A.a* and *P.g* in Atheromatose samples in patients with periodontitis, the author name, year of publication and the method of DNA detection are reported.

Reference	Method	n	Pg	Aa
Present study	RT-PCR	20	50	20
Gaetti-Jardim *et al.*, 2009 [17]	RT-PCR	44	53.8	46.2
Aquino *et al.* 2011 [25]	16S rRNA gene-based PCR	30	0	0
Figuero *et al.* 2011	Nested PCR	42	78.6	66.7
Calandrini, C, *et al.* 2014	16S rRNA gene-based PCR	35	-	20
Mahendra *et al.* 2014 [27]	16S rRNA gene-based PCR	51	45.1	0
Nakano *et al.* 2009 [28]	Specific PCR	223	20	30
Pyysalo *et al.* 2014 [16]	RT-PCR	36	<20	<20
Atarbashi-Moghadam *et al.* 2018 [29]	PCR	23	17.4	13

(Table 5) contd.....

Fernandes *et al.* 2014 [15]	RT-PCR	14	_	0
Mahalakshmi *et al.* 2017 [30]	PCR	65	0	52.3

CONCLUSION

The results of our research suggest a significant correlation between the quantities of *A.a* in subgingival and atherosclerotic plaque. However, the correlation between the quantities of *P.g* in subgingival and atherosclerotic plaque was not significant.

ETHICS APPROVAL AND CONSENT TO PARTI-CIPATE

This study was approved by the Ethics Committee of Mashhad Dental School, State University of Mashhad, Iran (under number IR.mums.sd.REC.139057).

HUMAN AND ANIMAL RIGHTS

No Animals were used in this research. All human research procedures were followed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

All the included subjects signed a consent form prior to the study.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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