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THE ASSOCIATION BETWEEN PERIODONTITIS AND CORONARY ARTERY ATHEROSCLEROSIS PATIENTS IN IRAQI POPULATION

AL-Nasrawy, Adel Ateyah
Genetic Engineering and Biotechnology Institute for Postgraduate studies-Baghdad University,
College of Dentistry - Kerbala University, Iraq.
Corresponding author: Adelmicro3000@gmail.com

ABSTRACT
This study was conducted to investigate the presence of a particular DNA from the most common periodontitis-associated bacteria *P. gingivalis* both at genus and species level in coronary artery atheromatous plaques retrieved by endarterectomy, This finding is provide an additional evidence that supports the potential association between chronic periodontitis and cardiovascular diseases, in which the periodontal *P. gingivalis* can access to the systemic circulation (bacteremia), colonize at distant sites, and thus, might influence the pathophysiology of coronary artery atherogenesis.

KEYWORDS: Coronary artery diseases: Atherosclerosis, Periodontitis, PCR; *Porphyromonas gingivalis 16S* ribosomal-RNA and *fimA* genotypes.

INTRODUCTION

Cardiovascular disease is a common cause of death in industrialized countries, accounting for 29% of deaths worldwide. Atherosclerosis is the principal cause of all cardiovascular diseases; it is responsible for 50% of all mortality in the United States, Europe, and Japan[1]. More than 50 prospective cohort and case control studies undertaken during the past 25 years demonstrated evidences for an association between periodontitis and cardiovascular diseases like, atherosclerotic vascular disease, including stroke, myocardial infarction, peripheral vascular disease, abdominal aortic aneurysm, coronary heart disease, and cardiovascular death [2,3]. Coronary heart disease is a narrowing of coronary arteries due to accumulation of fatty materials (plaque) build-up on its walls. This disease will decrease the oxygen-rich blood supply. Coronary heart disease patients will suffer from pain and discomfort known as angina [4]. Atherosclerosis is the major event in the pathophysiology of cardiovascular diseases, in which large- to medium-size muscular and large elastic arteries become occluded with fibrolipidic lesions, known as atheromas. These atheromatous plaque are responsible for end-stage complications or events associated with cardiovascular diseases, such as coronary thrombosis, acute myocardial infarction, and stroke [5]. Periodontitis is a chronic inflammatory disease caused by bacterial colonization, which results in destruction of the tissues between the tooth surface and gingiva, loss of connective tissue attachment, erosion of alveolar bone, and tooth loss, periodontitis is common and increases with age, in a United States of America survey, about half of adults aged >30 years have some periodontitis and almost 10% have severe disease [6].

Several studies have demonstrated that the presence of oral bacteria in atherosclerotic plaques is implicated in the pathogenesis of cardiovascular diseases, and that atherosclerosis and cardiovascular diseases are both accelerated by periodontal disease[3,7]. A link between periodontitis and cardiovascular disease has been proposed, periodontal disease and cardiovascular disease are highly prevalent in the modern community. Both pathologies are chronic inflammatory disorders, like periodontitis, atherosclerosis is a complex condition with a suspected microbial etiology in which *P. gingivalis* is attracting increasing attention for its possible role in accelerating disease progression[5,8]. *Porphyromonas gingivalis*, an anaerobic Gram-negative coccobacillus which belongs to the Bacteroidaceae family. In the natural environment, *P. gingivalis* is a constituent of the multispecies biofilm, It considered the main periodontal pathogen involved in onset and progression of various forms of periodontal diseases[9]. *P. gingivalis*, is a keystone pathogen in chronic periodontitis, it has been found to associate with remote body organ inflammatory pathologies, and it has the ability to evade the host immune response and access nutrients in the microenvironment which is directly related to its survival, proliferation, and infection. More recent analyses from large-cohort studies suggest new onset, and prevalent periodontitis, as well, is associated with increased cardiovascular diseases like Myocardial Infarction, Atherosclerosis[10], Diabetes Mellitus [11], Rheumatoid Arthritis[12], Preeclampsia with low birth weight[13], Orodigestive Cancers Mortality[14], and Alzheimer's Disease[15]. The systemic inflammatory or immune response to periodontal infection may increase cardiovascular risk. Also, pathogens from the mouth can enter atherosclerotic plaques via the blood stream, and this could promote an inflammatory or immune response within the atherosclerotic plaque. Adverse ranges of oral bacterial pathogens and bacterial DNA have been detected in atherosclerotic plaque [16]. Despite for heterogeneity of the studies, overall results of epidemiological studies suggest for a modest but significant association between periodontal infections and cardiovascular disease that is independent on the effects of confounders. Scientific

evidence supporting a possible role of oral bacterial species in atherosclerosis relies to a large extent on the detection and identification of bacterial DNA in human arterial wall tissues or atherosclerotic plaque in cross-sectional study designs[17]. DNA from *P. gingivalis*, a major periodontal pathogen, has been detected in coronary atherosclerotic plaques and atherosclerotic vessels[18].

An association between oral bacteria and atherosclerosis has been postulated. A limited number of studies have used *16S RNA gene* sequencing based metagenomics approaches to identify bacteria at the species level from atherosclerotic plaques in arterial walls [19]. Because of the high prevalence of periodontitis in humans, and because cardiovascular diseases are the main cause of death in developed countries, an increasing interest was raised in the scientific community to identify the potential links between both entities [20].

Therefore, the aim of this investigation was to detect DNA from periodontitis-associated bacteria *P. gingivalis* at the genus and species level in coronary artery atheromatous plaque recovered from patients using strict sample procurement and laboratory procedures. Our hypothesis was that bacterial DNA from periodontopathic bacteria would be present in the retrieved atherosclerosis samples, and this presence would be related to the oral health status of the patients.

MATERIALS &METHODS

Collection of Specimens

After clinical diagnosis of coronary artery atherosclerotic patients, atheromatous plaque thrombosis samples from diagnostic catheterization and therapeutic catheterization or both for seventy four coronary artery atherosclerotic patients (who received endarterectomies because of various manifestations of ischemic vascular disease) aged between 29 to 73 years who admitted to the Heart and Arteries catheterization Unit (Cardiology) in AL-Hussein Educational Hospital in Kerbala City during the period from July 2016 to April 2017. A pool of (24) diagnostic catheterization tissue specimens were taken from clinically non-atherosclerotic areas of coronary artery from subjects was obtained as a control group. Then, the atheromatous plaque samples were rapidly transferred into 1.5 ul polypropylene microcentrifuge tube contained 500 ul of 0.9% sterile normal saline solution, and subjected to the laboratory for molecular bacteriology detection.

TABLE 1: The Primers used in molecular detection of *P. gingivalis*

Gene	Duplexing primers 5´- 3´	Product size (bp)	Reference
P. gingivalis 16Sribosomal RNA	F AGG CAG CTT GCC ATA CTG CG R ACT GTT AGC AAC TAC CGA TGT	404	[23]
Type I *fimA*	F CTG TGT GTT TAT GGC AAA CTTC R AACCCC GCT CCC TGT ATT CCGA	392	[24]
Type Ib *fimA*	F CAG CAG AGC CAA AAA CAA TCG R TGT CAG ATA ATT AGC GTC TGC	271	[22]
Type II *fimA*	F ACAACTATACTT ATG ACA ATG G R AACCCCGCT CCC TGT ATT CCG A	257	[24]
Type III *fimA*	F ATTACACCTACA CAG GTG AGG C R AACCCCGCT CCC TGT ATT CCG A	247	[24]
Type IV *fimA*	F CTATTCAGG TGC TAT TAC CCA A R AACCCCGCT CCC TGT ATT CCG A	251	[24]
Type V *fimA*	F AACAACAGTCTC CTT GAC AGT G R TATTGG GGG TCG AAC GTT CTG TC	462	[25]

Amplification Reaction programs:-

TABLE 2: Cycling parameters for monoplex PCR of *16S rRNA* gene amplification

No. of cycles	Stage	Temperature °C	Time
1	Initial denaturation	95	5 min.
	Denaturation	94	30 Sec.
35	Annealing	60	30 Sec.
	Elongation	72	1 min.
1	Final extension	72	10 min.

TABLE 3: Cycling parameters for multiplex PCR of species specific *fimA* gene amplification

No. of cycles	Stage	Temperature °C	Time
1	Initial denaturation	95	5 min.
	Denaturation	94	30 Sec.
35	Annealing	58	30 Sec.
	Elongation	72	30 Sec.
1	Final extension	72	7 min.

Detection of *P. gingivalis* by Essential Genes

DNA Extraction

Isolation of DNA from atherosclerotic plaques samples were done using Genomic DNA Mini Kit (Geneaid, Korea) / Tissue using a protocol in accordance with the manufacturer's instructions, each atherosclerotic plaque sample (coronary artery plaque tissue of the catheter tip) with 500µl of 0.9% sterile normal saline was used for DNA extraction, extracted DNA aliquots were measured with Q5000 UV-Vis Spectrophotometer, 20-25 nanogram /microliter of extracted DNA aliquots were used for microbiological and molecular detection.

Molecular detection of *P. gingivalis* was performed by monoplex PCR of *16S rRNA* gene amplification according to [21] and multiplex PCR of species specific *fimA* gene amplification according [22]. Using the following amplification primers in (table 1) and according to the amplification reaction programs of (tables 2, 3).

Agarose Gel Electrophoresis

A concentration of (1, 2) % Agarose gel used for PCR products electrophoresis, which, accomplished with the use of two types of DNA ladder (Accu Ladder 100 bp Bioneer/Korea) and (50 bp DNA Step Ladder Marker Promega/ USA).

Statistical Analysis

The collected data were analyzed using the statistical system and Chi-Square ($\chi 2$) test, with P-value of ≤ 0.05.

RESULTS & DISSCUSION

3,000 years of history suggesting the oral influence, particularly of periodontitis on the general health of human subjects[26]. In periodontitis, *P. gingivalis* represents a keystone pathogen causing microbial and immune dysbiosis[27]. *P. gingivalis* has an arsenal of potent virulence factors, can invade periodontal, atherosclerotic, and brain tissue, thereby avoiding immune surveillance and maintaining its viability, it may act as the main organism in periodontitis and in related systemic diseases and other remote body inflammatory pathologies including dementia[15], atherosclerotic plaques of patients with cardiovascular diseases[16, 18]. More recent analyses from large-cohort studies suggest new onset, and prevalent periodontitis, as well, is associated with increased coronary heart disease risk[28] and there is a graded association between tooth loss and stroke, cardiovascular death, and all-cause mortality in patients with stable coronary artery disease[29].

The prevalence of the most Periodontal pathogen *Porphyromonas gingivalis* in the total 74 coronary artery atherosclerosis plaque Patients were 54/ 74 (73%) distributed in 36/ 54 (66.7%) in males and 18/ 54 (33.3%) in females and 33/ 54 (61%) in coronary artery atherosclerosis plaque Patients with 29-60 years and 21/ 54 (39%) in atherosclerosis plaque Patients with >60 years, more than in control group 5/ 24 (20.83%) as demonstrated in table no.1

TABLE 1. Distribution of Periodontal *Porphyromonas gingivalis* in coronary artery atherosclerosis plaque Patients and control groups

Variable		*Porphyromonas gingivalis* in Subject			
		Atherosclerosis group		Control group	
		(+)No, Percentage	(-) No, Percentage	(+) No, Percentage	(-) No, Percentage
Gender	Male	**36 (66.7%)**	12 (16.2%)	**3** (12.5%)	14 (58.33%)
	Female	**18 (33.3%)**	8 (10.8%)	**2 (8.33%)**	5 (20.83%)
Age	29-60 years	33 (61%)	14 (70%)	2 (8.33%)	13 (54.17%)
	> 60 years	21(39%)	6 (30%)	3 (12.5%)	6 (25%)

On the other hand, detection of *P. gingivalis* in coronary artery atherosclerosis plaque patients and control groups was achieved by monoplex PCR of *16S rRNA* gene amplification and multiplex PCR of species specific *fimA* gene amplification, all atherosclerosis plaque samples were positive for the genus specific level according to *16S rRNA* gene as demonstrated in figure no.1 and various species specific level according to *fimA* genotypes that were represented by *fimA* genotypes I, II, III, 1V and V as demonstrated in figures (2 and 3), these results indicate confirmatory diagnosis of the highly virulent periodontal pathogen *P. gingivalis* in atherosclerosis plaque samples of coronary artery disease patients, furthermore, interpret the ability of this potent bacterium to use its arsenal virulence factors in attaching different types of body tissues, establishing various complications, these results were agreed with multiple previous similar studies revealed that in addition to local inflammation at the initial site of infection, *P. gingivalis* has the ability to disseminate from ulcerative periodontal tissues to circulate and interact with the heart, liver, and other body tissues, therefore, *P. gingivalis* play an important role in periodontitis-associated systemic diseases, such as atherosclerosis[30].

Furthermore, presence of multiple *fimA* genotypes I, II, III, 1V & V in coronary artery atherosclerotic plaque samples in the present study is compatible to other scientific evidence supporting a possible role of oral bacterial species in atherosclerosis relies to a large extent on the detection and identification of bacterial DNA in human arterial wall tissues or atherosclerotic plaque in cross-sectional study designs[31]. Most importantly, Kozarov *et al.* demonstrated that viable *P. gingivalis* and *A. actinomycetemcomitans* could be isolated from atherosclerotic plaque[32].

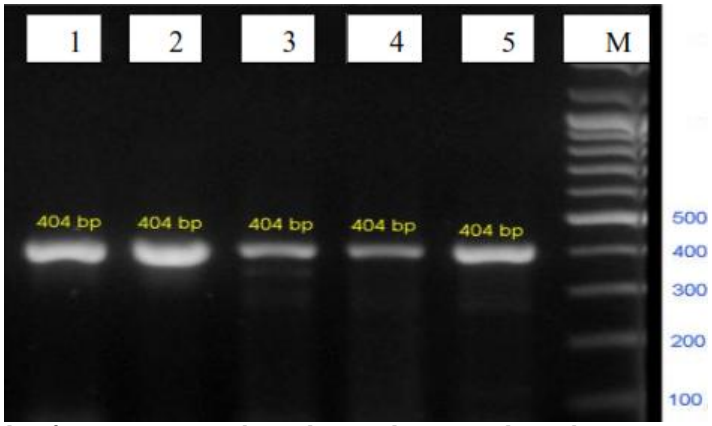

FIGURE 1: positive results of coronary artery atherosclerosis plaque samples with *16S rRNA* gene amplification. Lanes (1, 2, 3, 4, and 5): 404 bp. amplicon, and lane M: DNA 100 bp. molecular weight marker.

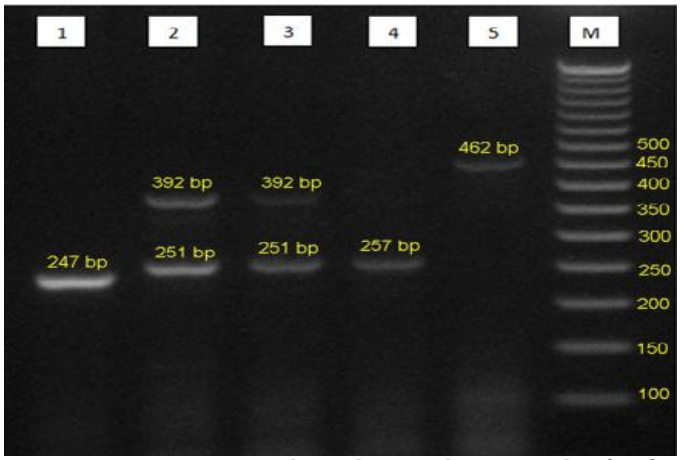

FIGURE 2: *P. gingivalis* positive coronary artery atherosclerosis plaque samples for *fimA* genotypes lane (1) *fimA* genotype (III) 247 bp., lanes (2, 3) *fimA* genotypes (1V) 251bp., and *fimA* genotype (I) 392 bp., lane (4) *fimA* genotype (II) 257 bp., lane (5) *fimA* genotype (V) 462 bp., and lane M= DNA Ladder (50 bp).

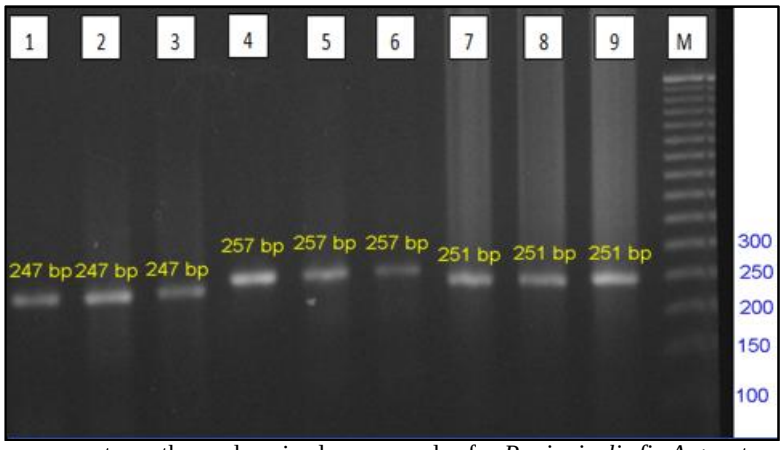

FIGURE 3: positive coronary artery atherosclerosis plaque samples for *P. gingivalis fimA* genotypes. Lanes (1, 2, 3) *fimA* genotype (III) 247 bp., lanes (4, 5, 6) *fimA* genotype (II) 257 bp., lanes (7, 8, 9) *fimA* genotypes (IV) 251 bp., and lane M= DNA Ladder (50 bp.).

The present study demonstrated the presence of DNA from periodontal bacteria *P. gingivalis* in atheromatous plaque retrieved from patients who received endarterectomies of various manifestations of ischemic coronary artery vascular diseases was 54/ 74 (73%), this prevalence was approximately to many previous investigations revealed that the prevalence of the most commonly periodontal virulent pathogen *P. gingivalis* in patients who received similar clinical cardiovascular manifestations was 33/ 42 (78.57%) in carotid artery atheromatous plaques samples[33], as well as previous clinical, epidemiological and molecular study indicated

that *P. gingivalis* was by far the most abundant species, representing nearly 80% of nearly 600 known oral bacterial species in artery tissues were obtained from patients with atherosclerotic cardiovascular disease who underwent coronary or femoral artery bypass surgery[34], and more than the concomitant detection of DNA from *P. gingivalis* observed in (61.90%) followed by *A. actinomycetemcomitans* (66.67%) of atheromatous samples[33]. As well many other authors identified *P. gingivalis* and *A. actinomycetemcomitans* as the most prevalent DNA from bacteria in atheromatous plaque from coronary arteries[35].

Another related study revealed a diverse range of oral bacterial pathogens and bacterial DNA has been detected in atherosclerotic plaque[16]. Etiologically, the chronic presence of periodontal microbes can lead to atherogenesis via two pathways: (1) direct invasion of the arterial wall and (2) the release, in response to infection, of systemic inflammatory mediators with atherogenic effects[36]. These pathogens, especially *P. gingivalis*, have demonstrated the ability to interact with the endothelial surface and to induce smooth-cell proliferation, causing damage and impairing the vasomotor functionality of the endothelial cells[37], indeed, in animal models, infection with *P. gingivalis* increases atherosclerotic plaque volume with the accumulation of cholesterol esters and inflammatory mediators[30,38]. In large cohort studies it has been suggested that pathogenesis of atherosclerosis is associated with both innate and adaptive immune responses. Maekawa *et al.*, 2011 claimed that oral infection with *P. gingivalis* accelerates atheroma formation by shifting the lipid profile of the host[39], and higher antibody titers against *P. gingivalis* have been detected in patients with cardiovascular disease and stroke[40], atherosclerosis [41], and myocardial infarction than in controls [42].

Furthermore, many (up to 30%) of coronary artery atherosclerotic plaque samples exhibited (2-3) *fimA* genotypes in the same site and single *fimA* genotype in the control group enrolled in the current study, these investigations were suggested various explanations, such as the presence of several different *P. gingivalis fimA* genotypes colonizing the same atherosclerotic site, and a higher intra individual heterogeneity of *P. gingivalis* which established and showed allelic variation in the *P. gingivalis* housekeeping genes indicating genetic recombination and genetic variability, resulted different clones of *P. gingivalis fimA* genotypes colonizing the same atherosclerotic plaque site as demonstrated in *P. gingivalis fimA* genotypes colonizing the same periodontal pocket[43, 44]. Other investigation demonstrated that the recognition of genes linked to chromosome 9p21, and related to transforming growth factor beta regulation, predisposes to periodontitis, and to coronary artery disease, as well, provides further evidence that common pathophysiological pathways are important for the two diseases [45]. Indeed, previous support study indicated that severe periodontal disease is associated with a 25% to 90% increase in risk for cardiovascular diseases after adjustment of other risk factors [46], if causal, these associations would be of great importance because of the potential that preventing or treating periodontal disease could reduce the risk of major adverse cardiovascular events [3].

CONCLUSIONS & RECOMMENDATION

Within the limitations of this investigation, we have identified periodontitis-associated bacterial DNA in coronary artery atheromatous plaque retrieved by endarterectomy, These findings provide additional evidence that supports the potential association between periodontitis and cardiovascular diseases, in which a keystone periodontal *P. gingivalis* which access to the systemic circulation (bacteremia), colonize at distant sites, and thus, might influence the pathophysiology of atherogenesis. However, the mere presence of bacterial DNA in this atheromatous plaque did not imply that live bacteria were present within the plaque, and therefore, further investigations are warranted. These studies should seek microbiologic data from atheromatous plaque and gingival crevicular fluid (GCF) and serum from the same patients, thus being able to confirm this likely direct relationship between periodontitis and cardiovascular diseases.

Confirmatory studies are thus needed to determine the number and abundance of more virulent pathogenic species present in atherosclerotic plaque and clinically periodontitis patients for activation of vaccination programs or protocols in order to minimize or get rid of these two chronic, problematic, related dangerous syndromes.

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