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RESEARCH PAPER

Prevalence of periodontal pathogens in coronary atherosclerotic plaque of patients undergoing coronary artery bypass graft surgery

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Abstract
Background Chronic bacterial infections have been associated with an increased risk for atherosclerosis and coronary artery disease. The ability of oral pathogens to colonize in coronary atheromatous plaque is well known. The aim of our study was to detect the presence of four common periodontal pathogens in coronary plaques. We detected the presence of 16S rRNA of *Treponema denticola, Eikenella Corrodens, Porphyromonas gingivalis* and *Campylobacter rectus* in subgingival and atherosclerotic plaques of CABG surgery by using Polymerase Chain Reaction.
Methods 51 patients in the age group of 40 to 80 years with chronic periodontitis were recruited for the study. These patients were suffering from Coronary Artery Disease (CAD) and underwent Coronary Artery Bypass Grafting (CABG). DNA was extracted from the subgingival plaque and coronary atheromatous plaque samples. Universal Primer for the general detection of bacterial DNA and the primers for *T.denticola, E. Corrodens, C.rectus* and *P.gingivalis* were used to amplify part of 16SrRNA gene by Polymerase Chain Reaction.
Results *T.denticola, E.corrodens, C.rectus* and *P.gingivalis* were detected in 49.01 %, 27.45 %, 21.51% and 45.10% of atherosclerotic plaque samples . In both subgingival and coronary plaque samples, *T. denticola* was detected in 39.21% of the cases, *E.corrodens* in 19.60%, *C.rectus* in 11.76% and *P.gingivalis* in 39.22% of the cases respectively .
Conclusion Our study revealed the presence of significant bacterial DNA of oral pathogens in coronary plaques. This suggests possible relationship between periodontal infection and atherosclerosis and can help devise preventive treatment strategies.

Keywords Atherosclerosis · Periodontal diseases · Periodontal pathogens · Inflammation and atherosclerosis · Polymerase chain reaction

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Introduction

Atherosclerosis is known to develop in response to vessel wall injury [1]. Damage to the endothelium is the main cause for development of the atherosclerotic plaque which is an inflammatory and fibro proliferative response to a variety of insults. Inflammation constitutes a major factor in the development of atherosclerosis and plaque disruption followed by local thrombosis being responsible for the clinical presentation of acute coronary syndromes [2–4]. The classical risk factors like hypercholesterolemia, cigarette smoking, diabetes mellitus and hypertension do account for majority of the aetiology, pathogenesis and clinical manifestations of atherosclerosis, including ischemic heart disease and acute myocardial infarction [5].

Infectious agents were implicated in atherosclerosis in the beginning of this century [6,7]. However, it was not until the late 1970s, when Fabricant et al. [8] showed that chickens experimentally infected with an avian herpes virus developed florid vascular lesions similar to those of human atherosclerosis. Subsequently, many investigators have reported observations implicating *Chlamydia pneumoniae, Helicobacter pylori, Herpes simplex virus,* and *Cytomegalovirus* as possible primary etiologic factors or cofactors in the pathogenesis of atherosclerosis, including ischemic heart disease and cerebrovascular disease [9–14].

Two basic lines of evidence suggesting a role for inflammation in the development of atherosclerosis have been proposed. (1) detection of the microorganisms in coronary atherosclerotic lesions by immunocytochemistry or molecular biology and (2) epidemiological evidence based on serological data implicating an

association between coronary atherosclerotic disease and positive serology [15].

Coronary Artery Disease (CAD) and periodontal diseases are common inflammatory conditions in human population [16]. Chronic infections have been implicated as an increased risk factor for coronary atherosclerosis [17]. CAD is one of the leading causes of premature death in the adults. Coronary atherosclerosis is the most frequent cause of CAD and plaque disruption with superimposed thrombus is the main mechanism of myocardial infarction. A key role for inflammation has been established, suggesting that inflammatory processes underlie all phases of coronary atherosclerosis, from the initial formation of plaques to their progression and rupture, which lead to clinical events such as unstable angina, acute myocardial infarction, and sudden death [2]. Oral surgical procedures may lead to bacteremia which may produce bacterial endocarditis and other systemic complications. Anaerobic bacteria have been detected in human blood under a variety of conditions including bacteremia after oral surgery [18]. Similarly, the periodontal infection is a chronic infectious disease characterized by inflammatory changes in periodontal tissues. The disease occurs as a result of infection associated with a small number of predominantly Gram-negative micro organisms and spirochetes [19]. Oral gram-positive and gram-negative bacteria have frequently been identified in bacteremia and may play a role in vascular diseases. Recent epidemiological studies suggest that periodontal disease may be an important risk factor for CAD. Various case control studies have shown a significant association after correction for conventional risk factors like smoking, hypertension, obesity and dyslipidemia [20]. Possible mechanism could be endothelial injury by oral microbial toxins and systemic inflammation triggered by oral infections. In addition phagocytes in periodontal lesions may engulf various bacterial cells and their antigens. The bacterial cells and phagocytes may then penetrate the gingival tissues and get transported through circulation to the heart, and adhere to the coronary artery endothelium. These deposited bacteria can then stimulate the release of inflammatory cytokines and initiate the formation of the characteristic foam cells associated with atherosclerosis [21].

The aim of our study was to detect the presence of four common periodontal pathogens in atherosclerotic plaques and subgingival plaques. We detected the presence of 16SrRNA of *Treponema denticola, Eikenella Corrodens, Porphyromonas gingivalis* and *Campylobacter rectus* in subgingival plaque and atherosclerotic plaques of patients undergoing Coronary Artery Bypass Grafting (CABG) by using Polymerase Chain Reaction (PCR) test.

Material and methods

Patients selection

51 patients (11 females and 40 males) in the age group of 40–80 years from 1st November 2007 to 30th November 2008 with chronic periodontitis were recruited consecutively from the Institute of Cardiovascular disease, Madras Medical Mission, Chennai. These patients were suffering from CAD and were scheduled to undergo CABG. Exclusion criteria included major systemic illness, advanced malignancy, antibiotic intake and periodontal treatment in the previous 6 months.

The medical and dental history of each subject was obtained by an interview. Patients fulfilling the inclusion criteria were selected for the study and an informed consent was obtained from them. The ethics committee of Madras Medical Mission approved the protocol of this study.

Collection of samples from the subgingival and atheromatous plaques (Fig. 1)

Subgingival plaque sample

The samples were taken one day before the patients underwent CABG. A periodontal examination was performed by the Dental Surgeon. Clinical evaluation included plaque index, probing depth, periodontal index, total number of teeth present and clinical attachment loss. The deepest periodontal sites with periodontal depth ≥5 mm were selected for the microbial sampling. The teeth were gently dried with sterile cotton swab. After the removal of supragingival plaque, the subgingival plaque samples were obtained with the help of curette from the two deepest periodontitis sites and were pooled for analysis.

Coronary atheromatous plaque sample

A biopsy was obtained from the coronary atherosclerotic plaque during the CABG. The surgeon excised one or two small bits of plaque (0.5 to 1 mm) from the edge of the coronary arteriotomy performed for anastomosing the graft. To eliminate blood contamination, the samples were placed in sterilized phosphate buffered saline and mixed gently and tissue samples were transferred to fresh vials containing the transport media. The samples were then homogenized by the tissue homogenizer as described by Saiki et al. 1988 [22].

DNA extraction

Both the subgingival plaque and coronary atherosclerotic plaque samples were centrifuged for 10 minutes at 10,000 rpm. The supernatant fluid was discarded and the resulting pellet was re-suspended in 200 µl of lysis solution (100 mm Tris, 1.0 mm EDTA, 1.0% Triton x –100 pH – 7.8). The sample was kept in the boiling water bath for 10 minutes, allowed to cool and again

PCR primers of the microorganisms in the study are as listed below.

Microorganisms	Product size
Campylobacter rectus TTT CGG AGC GTA AAC TCC TTT TCTTT CTG CAA GCA GAC ACT CTT	415-1,012 (598)bp
Eikenella corrodens CTA ATA CCG CAT ACG TCC TAA GCTA CTA AGC AAT CAA GTT GCC C	169-856 (688)bp
Porphyromonas gingivalis AGG CAG CTT GCC ATA CTG C ACT GTT AGC AAC TAC CGA TGT	729-1,132 (404)bp
Treponema denticola TAA TAC CGA ATG TGC TCA TTT ACA TTCA AAG AAG CAT TCC CTC TTC TTC TTA	316bp
Ubiquitous primer (universal primer for 16s RNA of bacterial species) GAT TAG ATA CCC TGG TAG TCC AC CCC GGG AAC GTA TTC ACC G	602bp

To explore the association between the presence of Treponema denticola, Eikenella corrodens, Campylobacter rectus and Porphyromonas gingivalis in both the plaque samples with the periodontal parameters, correlation coefficient was calculated and are shown in Table 1 and 2 respectively.

Table 1 Correlation coefficient analysis of Treponema denticola and Eikenella corrodens

Periodontal parameters	Treponema denticola		Eikenella corrodens	
	Detected in subgingival plaque	Detected in coronary atherosclerotic plaque	Detected in subgingival plaque	Detected in coronary atherosclerotic plaque
Total no. of teeth	-0.0073 NS	-0.0580 NS	-0.0638 NS	0.0112 NS
Plaque index	-0.0051 NS	0.336*	0.3697**	0.2845*
Gingival index	-0.0096 NS	0.0197 NS	0.0415 NS	0.2499 NS
Russels periodontal index	0.0352 NS	0.1869 NS	0.3212*	0.2924*
Clinical attachment level	0.2614 NS	0.3382*	0.5599**	0.2846*
Probing depth index	0.1083 NS	0.3212*	0.4972**	0.2820*

NS - Non Significant (P >0.05)
* - Significant (P≤0.05)
** - Highly significant (P≤0.01)

Table 2 Correlation coefficient analysis of campylobacter rectus and porphyromonas gingivalis

Periodontal parameters	Campylobacter rectus		Porphyromonas gingivalis	
	Detected in subgingival plaque	Detected in coronary atherosclerotic plaque	Detected in subgingival plaque	Detected in coronary atherosclerotic plaque
Total no. of teeth	0.0931	0.2764*	0.2147	0.0792
Plaque index	0.2847*	0.2118	0.5455**	0.3809**
Gingival index	-0.0841	-0.0671	0.1123	-0.1234
Russels periodontal index	0.1613	0.1331	0.4574**	0.3799**
Clinical attachment level	0.1217	0.0438	0.2570	0.2252
Probing depth index	0.2076	0.1059	0.3207*	0.2841*

NS - Non Significant (P >0.05)
* - Significant (P≤0.05)
** - Highly significant (P≤0.01)

centrifuged for 5 minutes at 10,000 rpm. Supernatant fluid was collected as DNA Template and stored at -70°C.

PCR amplification

16SrRNA PCR amplification was carried out to detect the presence of microorganisms. PCR Primers used in the study, were designed as per Larsen et al. (1993) [23].

The upstream and down stream sequence primers were then verified for their species specificity by comparing the sequences with all available 16SrRNA sequences in the RDP database. Ubiquitous Primer was used as the positive control for PCR amplification. PCR was performed as described by Saiki et al. (1988) [22]. 10 µl of DNA (The specific quantity of DNA i.e. 10 µl of DNA has been incorporated. 1 Microlitre = 0.000001 Litres). Template of the sample was added to 40 µl of working stock reaction mixture containing 5 µl of 10 x PCR buffer, 1.25 unit of Taq DNA Polymerase (0.4 µl) , 0.2 mM (1µl) of each deoxyribonucleotides (dNTP's), Primers (1 µl) Forward and (1 µl) reverse of the specific microorganisms and 31.6 µl of milli Q water. The PCR reaction was carried out using a PCR thermocycler, Applied Biosystems (USA). The PCR temperature profile for all the four microorganisms included an initial denaturation of 95° C for 2 minutes, followed by 36 cycles of denaturation step at 95° C for 30 seconds, annealing step at 60° C for 1 minute, extension at 72° C for 1 minute and final step at 72° C for 2 minutes.

After amplification, 10µl aliquot of amplified PCR product was subjected to electrophoresis in a 0.75 % agarose gel containing 0.5 µg/mL^{-1} Ethidium bromide in 1 x TAE buffer. The gel was photographed under 300 nm, ultraviolet light transilluminator. A 100 bp DNA ladder (Bangalore Genei Pvt Limited) served as a molecular weight marker (BIORAD). The PCR amplified products were sequenced in an automated sequencer (Genetic analyzer 3130, Applied Bio Systems, USA). The sequencer data was blasted with available data in Gen Bank and compared for possible homologies. The product size has been standardized as per Ashimoto et al (1996) and Larsen et al (1993).

The detection of the oral pathogens namely *Treponema denticola, Eikenella Corrodens, Porphyromonas gingivalis* and *Campylobacter rectus* in the subgingival plaque, the coronary atherosclerotic plaque and in both locations are shown in Figs. 1a, 1b, 1c, and 1d respectively.

Results

Treponema denticola, Eikenella corrodens, Campylobacter rectus and *Porphyromonas gingivalis* were detected in 66.66%, 47.06%, 29.41% and 64.71% of subgingival plaque samples and in 49.01%, 27.45%, 21.51% and 45.10% of atherosclerotic plaque samples. In both subgingival plaque and coronary atherosclerotic plaque samples Treponema denticola was detected in 39.21%, Eikenella corrodens in 19.60%, Campylobacter rectus in 11.76% and Porphyromonas gingivalis in 39.22% respectively. 9.80% of Treponema denticola, 7.85% of Eikenella corrodens, 9.75% of Campylobacter rectus and 5.88% of Porphyromonas gingivalis was found only in coronary atherosclerotic plaque without the presence of these microorganisms in subgingival plaque.

Porphyromonas gingivalis

Results revealed that there was a significant correlation between the plaque index and presence of Treponema denticola in atherosclerotic plaque ($P \leq 0.05$). There was also significant correlation between the *Treponema denticola* in atherosclerotic plaque with clinical attachment level and probing depth ($P \leq 0.05$) (Table 1).

There was statistically significant correlation between E.Corrodens in atherosclerotic plaque with plaque index, Russels periodontal index, clinical attachment level and probing depth ($P \leq 0.05$). There was also a highly significant association ($pd \leq 0.01$) of the microorganism in the subgingival plaque with plaque index,clinical attachment level and probing depth and significant association ($pd \leq 0.05$) with Russel's periodontal index (Table 1).

There was a statistically significant association between the C. rectus in atherosclerotic plaque and subgingival plaque with the total number of teeth and plaque index respectively (Table 2).

There was a highly significant association between plaque index and Russel's periodontal index for the presence of P. gingivalis in subgingival and atherosclerotic plaque. ($P \leq 0.01$). There was a statistically significant association between probing depth and P. gingivalis in both the plaque samples ($P \leq 0.05$) (Table 2).

The above observations reveal that the amount of periodontal destruction directly correlates with the presence of the four periodontal microorganisms in coronary atherosclerotic plaque samples.

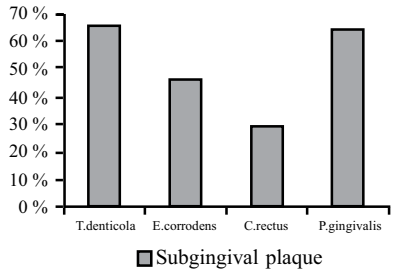
Chi-square analysis

Chi-square analysis was carried out to find out the significant difference between the four microorganisms in subgingival plaque and coronary atherosclerotic plaque samples. It was found that the value of chi-square was significant and hence variables were dependent.

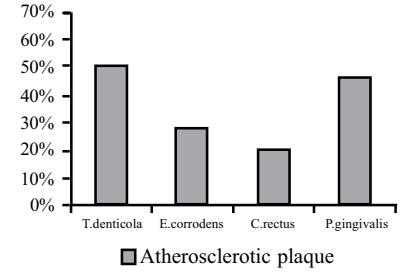
Discussion

A number of epidemiological studies have shown a statistical association between periodontitis and CAD. Presence of oral pathogens in coronary atherosclerotic plaques have been reported by Haraszthy et al. [24].

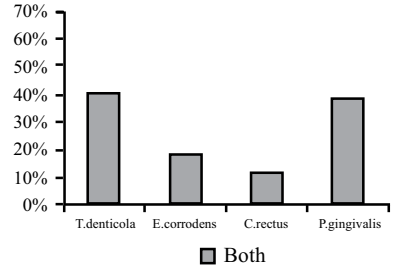
The incidence of periodontal pathogens in subgingival plaque, atherosclerotic plaque and both sites are demonstrated in bar diagrams a, b, c, d as depicted below.



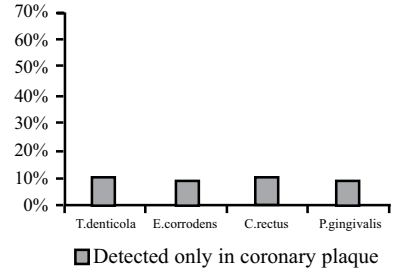
a) Prevalence of oral pathogens in subgingival plaque



b) Prevalence of oral pathogens in coronary atherosclerotic plaque



c) Prevalence of oral pathogens in subgingival plaque and coronary atherosclerotic plaque



d) Prevalence of oral pathogens only in coronary atherosclerotic plaque

Periodontitis and atherosclerosis have many pathogenic mechanisms in common. Both the diseases have complex causation, genetic and gender predisposition and might share many risk factors, such as age, education, smoking, social status, and stress [25].

Our study was done with the aim to investigate and compare the presence of four common oral pathogens namely *Treponema denticola, Eikenella corrodens, Campylobacter rectus* and *Porphyromonas gingivalis* in coronary atheromas recovered from patients undergoing CABG. Coronary atheromas and subgingival plaque samples were collected in 51 patients and analyzed for universal bacterial primers followed by the specific primers for these microorganisms. 16SrRNA specific sequence of the above microorganisms were selected. Hence, the presence of both bacterial DNA and the microorganisms could be ascertained. Both the subgingival plaque and coronary atherosclerotic plaque samples revealed bacterial DNA. However, the prevalence of *Treponema denticola, Eikenella corrodens, Campylobacter rectus* and *Porphyromonas gingivalis* was detected in 66.66%, 47.06%, 29.41% and 64.71% of subgingival plaque samples and in 49.01 %,

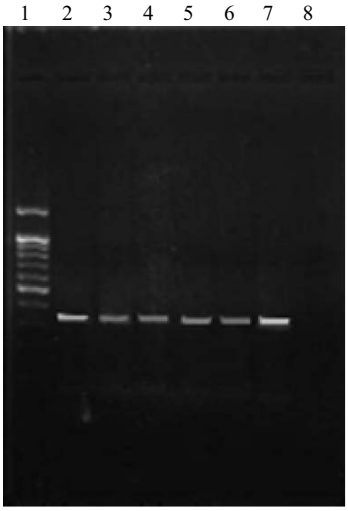


Fig. 1a 16SrRNA-based PCR detection of Treponema denticola
Lane 1: DNA ladder–100 bp
Lane 2, 4 & 6: Subgingival plaque samples
Lane 3, 5 & 7: Coronary artery plaque samples
Lane 8 : Negative control

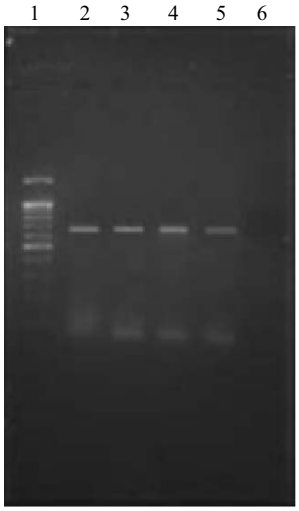


Fig. 1b 16SrRNA-based PCR detection of Eikenella corrodens
Lane 1: DNA ladder–100 bp
Lane 2 & 4: Subgingival plaque samples
Lane 3 & 5: Coronary artery plaque samples
Lane 6: Negative control

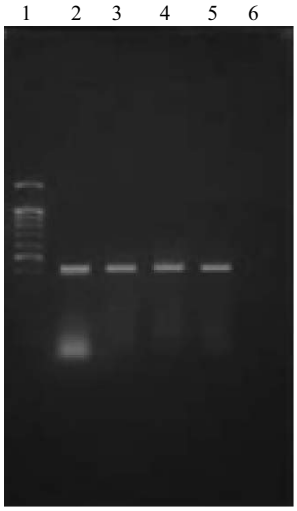


Fig. 1c 16SrRNA-based PCR detection of Porphyromonas gingivalis
Lane 1: DNA ladder–100 bp
Lane 2 & 4: Subgingival plaque samples
Lane 3 & 5: Coronary artery plaque samples
Lane 6: Negative control

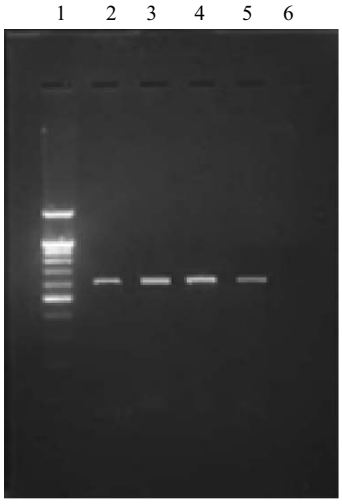


Fig. 1d 16SrRNA-based PCR detection of Campylobacter rectus
Lane 1: DNA ladder–100 bp
Lane 2 & 4: Subgingival plaque samples
Lane 3 & 5: Coronary artery plaque samples
Lane 6: Negative control

27.45 %, 21,51% and 45.10% of coronary atherosclerotic plaque samples respectively. In both subgingival plaque and coronary atherosclerotic plaque samples *Treponema denticola* was detected in 39.21%, *Eikenella corrodens* in 19.60%, *Campylobacter rectus* in 11.76% and *Porphyromonas gingivalis* in 39.22% respectively. 9.80 % of *Treponema denticola,* 7.85 % of *Eikenella corrodens,* 9.75% of *Campylobacter rectus* and 5.88% of *Porphyromonas gingivalis* was found only in coronary atherosclerotic plaque without the presence of these microorganisms in subgingival plaque.

Treponema denticola is a gram-negative bacterium from the Spirochetes family that is motile, slender, helically shaped and flexible. The organism consists of periplasmic flagella, which allows for mobility by using a proton motive force to cause thrusting through rotation. It is commonly found in the human oral cavity, specifically in subgingival dental plaque, and it is often associated with periodontal disease. The organism causes disease by aggregating in subgingival plaque with Porphyromonas gingivalis and it uses several mechanisms in order to survive harsh conditions, such as oral biofilms [26].

Eikenella corrodens is a facultative gram-negative bacillus, which is a common inhabitant of the oral cavity, intestinal and genital tracts. It is often present in the supra and subgingival plaque of periodontally healthy subjects. It appears to be somewhat more prevalent in subgingival plaque samples of patients with periodontitis than healthy individuals [27].

Porphyromonas gingivalis is a black pigmented gram negative anaerobic coccobacillus which is one of the major pathogen implicated in chronic periodontal disease. It is known to express various pathogenic factors such as fimbriae, adhesins, lipopolysaccharides, enzymes like collagenase and protease [20].

Campylobacter rectus, a gram-negative, microaerophilic, and motile bacterium, has been proposed to play a pathogenic role in human periodontitis. Surface components, such as the flagellum, surface layer (S-layer), and cytotoxin, have been reported as possible virulence factors of the microorganism.

Recently studies have shown the variations in detection of periodontal pathogens in subgingival and atheromatous plaques using PCR assays. Cairo et al. [28] and Ishihara et al. [29] reported the presence of atleast one target bacterial species in 75% of the samples. Our results have shown that oral pathogens detected in the periodontal sites are also detected with a higher prevalence in the diseased coronary artery. This is in accordance with the study by Aimetti et al. [30] which showed the prevalence of *T. denticola* in subgingival plaques and atherosclerotic lesions as 54.5%. With the set of ubiquitous primers for the general detection of bacterial DNA, all the subgingival and atherosclerotic plaque samples were positive for the bacterial DNA. This proportion is greater than that reported by Aimetti et al. [30] and Haraszthy et al. [24] and is similar to that presented by Fiehn et al. [31] i.e.100%.

We detected bacterial DNA of the above four microorganisms in the diseased coronary atherosclerotic arteries by 36 amplified cycles. According to the severity of the disease, these four microorganisms may enter the circulation more easily and can subsequently get colonised in the coronary atherosclerotic plaque.

The chronic cyclic nature of periodontal disease provides multiple opportunities for repeated dissemination of pathogens in the blood. This can also explain the absence of organisms in the subgingival plaque when it is detected in the coronary plaque. Gingival ulceration and vascular changes in the periodontal tissues increase the incidence and

severity of transient bacteremia. The bacteremia could affect the vascular endothelial integrity, metabolism of plasma lipoproteins, blood coagulation and platelet function [32].

Conclusion

Our study revealed the presence of bacterial DNA of periodontal pathogens in coronary atherosclerotic plaques. Their presence in significant proportion may suggest the possible relationship between periodontal bacterial infection and genesis of coronary atherosclerosis. This observation can provide insights into the pathogenesis of coronary atherosclerosis and help to devise preventive treatment strategies including primary oral prophylaxis for this life threatening disease.

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