Invasion of Diabetic and Atherosclerotic Vessels Patients with Periopathogen Spirochete *Treponema denticola* in Iraqi Population

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Abstract

Both Periodontitis & Atherosclerosis of Cardiovascular system are chronic inflammatory disorders can be performed by invasion of their connective tissues with aerobic & anaerobic infectious bacteria & formation of polymicrobial biofilms. Microbial & metabolic plaques are considered to be the initiating factor for many infectious & metabolic diseases, invasion of an oral & systemic organs with various opportunistic pathogens have been reported in many populations. Several studies have been published Conventional & Real time PCR with *16S r RNA gene* to improve the existence of the DNA molecules of the most invasive periodontics pathogens in atheromatous plaques of diabetic & cardiovascular blood vessels disease patients.

In the present study, among 100 atherosclerotic & diabetic patients suffered from various degrees of ischemic heart disease, the DNA molecules of *Treponema denticola* was detected in (45/100)45% of atheromatous plaque samples significantly in male 28 /45 (62%) more than 17/45(38%) in female patients (p = 0.07) with no significant importance in cardiovascular & diabetic patients more than 50 years old 23/45(51%) which was approximate to 22/45(49%) in younger than 50 years old. As well as, *T. denticola* DNA molecules were significantly displayed in atheromatous plaques of type (1, 2) diabetic patients 35 /45 (78%) more than in non-diabetic group 10/45(22%) (p= 0.07) & *T. denticola* DNA molecules did not displayed in the controller group.

In conclusion: the present cross sectional study demonstrated the invasion of atheromatous plaques of cardiovascular atherosclerotic vessels & diabetic patients with highly aggressive anaerobic periodontal spirochetes *T. denticola*, these results improve the existence of additional risk factor that interfere with the pathogenesis of periodontitis & genesis of cardiovascular atherosclerosis & may be correlated with the atherosclerosis & diabetes mellitus diseases patients in Iraqi population.

Keywords: *Periodontitis, Atherosclerosis Biofilms, Spirochete Treponema denticola, Serotype-specific primers of 16S ribosomal-RNA gene and PCR.*

Introduction

Cases of diabetes is severely growing internationally which characterizes an significant problem for patients & for the society as well due to micro & macro vascular complications that people with this condition may experience & consequently cardiovascular diseases that are the most predominant causes of illness & death among patients with diabetes. Cardiovascular diseases (CVD), which internationally rank 1st in the list of illness & death, are public in many adult populations over the last 15 years. Atherosclerosis is the main fundamental vascular disease responsible for cardiovascular & cerebra vascular death & illness. The development of atherosclerosis & the generating of cardiovascular proceedings includes the interference of a sequence of risk factors, such as age, smoking, hypertension, diabetes mellitus, insulin resistant & hypercholesterolemia [(1)] . Atherosclerosis is the progressive build-up of plaque, & biomass creation which consists of a mixture of the macrophages, lipids, smooth muscle cells, platelets, clotting factors, & the products of infection, including antigens, antibodies, & various immune complexes. [(2)] .

Atherosclerotic CVD, previously the primary source of passing away in the USA , is predicted to be the most common cause of death in all parts of the world by the year 2020 [3] . Connotation between infectious disease, atherosclerosis, & cardiovascular disease was described as initially in 1911 [4] . Bacterial & /or viruses infection have been hypothetically to possibly contribute to the pathogenesis of atherosclerosis through direct & indirect mechanisms. Numerous quantity of Periopathogen have been described in cardiovascular atheromatous plaques according to the environment throughout the world, most of them have been proposed to have associations with cardiovascular diseases [5, 6] . Pathogenesis of the disease prompted by infectious agents has been refer to 3 diverse ways of act : discharge of toxins or super antigens, induction of inflammation, & molecular mimicry or cross-reactivity. Any of these ways can product in plaque foundation [7] .

Periodontitis is a chronic inflammatory disease of the connective tissues surrounding the teeth, significantly, one of the main causes of the progress & development of periodontal disease is characterized by bloated of the concentration of particular Periopathogen within the sub gingival plaques that stimulates a massive noxious immune response [8] . Chronic Periodontitis caused by specific anaerobic pathogens, marks advises that periodontal infection may significantly increase the risk for certain systemic conditions including atheromatous plaques of coronary heart disease with related events such as angina & myocardial infarction, atherosclerosis, stroke, diabetes mellitus, preterm labor, low birth-weight delivery, & respiratory conditions. [9] Several studies have reported epidemiological associations between periodontitis & cardiovascular diseases [10] .

Treponema denticola is a Gram-negative, slender, helically shaped & flexible, anaerobic motile bacteria from the spirochetes family, it shows ordinarily four periplasmic flagella, which facilitate its mobility even in a viscous environment [11] it is part of the microbiota of the human oral environment & exists as one member of the red complex bacteria (*T. denticola, Porphyromonas gingivalis, as well as Tenerella forsythia*) that has been prominently participate in severity & complexity of chronic periodontitis & strongly associated with polymicrobial biofilms formation in oral periodontal lesions & it initiates dysregulation of inflammation & tissue homeostasis [12] . *T. denticola* have major virulence factors in chronic periodontitis & other systemic infections represented by its motility & chemotaxis, which enable the bacterium to rapidly colonize new sites, penetrate deep periodontal pockets, & penetrate epithelial layers as well as its ability to produce cytotoxic metabolites & a range of cell-surface proteins for *in vivo* biomass & polymicrobial biofilms formation to dysregulate the host defense mechanisms & cause host tissue destruction [13] as well as *in vitro* biofilm formation [14] .

During the last two decades, detection & quantification of DNA molecules of periodontal pathogens was differ in the frequency among the reported studies, several cross sectional & epidemiological studies have been published Real time PCR & conventional PCR with *16 Sr RNA* gene to improve the presence of DNA molecules of Periopathogen in periodontal plaques [15,16] & in atheromatous plaques of cardiovascular system [17, 18, 19, 20]. Therefore, the aim of the present study was to demonstrate the presence of periodontal pathogen spirochetes *Treponema denticola* in atheromatous plaques of Iraqi cardiovascular diseases & diabetic patients & sustain the role of this putative risk factor in chronic systemic infections.

Materials & Methods

Work was done from October 2018 to December 2019; Ethical clearance was obtained from the Ethical Committee of Ministry of Health in Kerbala Health organization. Gathering of athermanous plaque specimens was performed in Unit of Cardiology of AL-Hussein Educational Hospital in Kerbala City, (100) Cardiovascular diseases patients (63 males & 37 females) were suffered from various degrees of ischemic heart disease, as well (35) of them have type (1, 2) diabetes mellitus & periodontitis was diagnosed in more than 50% of the patients were enrolled in this study, all patients were aged between (38 to 74) years old who received the surgical procedures of catheter-based atherectomy, endarterectomy because of several

manifestations of ischemic heart valve, coronary artery & blood vessels atherosclerotic patients, as well as a group of (20) diagnostic catheterization tissue samples were gotten as a control group. Indeed, written informed consent (questioner) was obtained from the subjects immediately with the samples collection.

Collection of Specimens & DNA Isolation

Vascular tissues & atheromatous plaque biopsies were collected under the surgical treatment from the walls of aneurysms & during excision of intravascular plaques from concerned blood vessels such as coronary artery & common carotid artery & others have been taken from the tips of the therapeutic & diagnostic catheters of CVD patients & control group were achieved with highly sterile conditions, each endarterectomy specimen was immediately transferred into1.5 ul polypropylene microcentrifuge tube 500 ul of 0.9 % disinfected normal saline solution , quickly moved to the research laboratory for molecular examination.[59-62] .

Genomic DNA Extraction

Total DNA samples were extracted from all of the vascular & atheromatous plaque biopsies by using Tissue protocol DNA Purification Mini Kit (Genaid, Korea) conferring to the producer's extraction process. The quantification of genomic DNA was measured with Q5000 UV-Vis Spectrophotometer at (260nm) & DNA quality was measured by the 260:280 nm absorbance ratio, & about 20 – 25 nomogram / microliter of isolated DNA cast-off for uncovering of *Treponema dentocola* by Polymerase Chain Reaction (PCR) technique under strict aseptic conditions & according to [21] .

Molecular Detection of *Spirochetes Treponema denticola By* PCR

Purified DNA samples were used for detecting the genus & species of *T. denticola* by PCR depending upon Species-specific pair primers for *16S ribosomal RNA* gene of *Treponema denticola* that demonstrated in table no.1, according to the amplification reaction program in table no.2. in accordance to [21, 22] techniques.

Table 1: Primer sets used for detecting of *Treponema denticola* by PCR.

Bacterium	Sequence of Primers (5 – 3)		Length
Treponema denticola	F	5 " – T A A T A C C G A A T G T G C T T T A C A T - 3 "	316 bp
	R	5 " - T C A A A G A A G C A T T C C C T C T T C T T C T T A - 3 "	

Table 2 : (Amplification reaction program) for amplifying 16 S r R N A gene of the Genus & Specie-specific of Treponema denticola by PCR technique [21, 22, 23] .

No. of cycles	Stage	Temperature °C	Time
1	Initial denaturation	95	2 min.
36	Denaturation	95	30 Sec.
	Annealing	60	1 min.
	Elongation	72	1 min.
1	Final extension	72	2 min.

DNA Analysis & Electrophoresis

Amplified DNA products of PCR reaction for Atherosclerotic & diabetic plaques & control specimens were analyzed by electrophoresis in a concentration of (1.5) % Agarose gel , separation of the amplified DNA product was done by mixing 5 µl of PCR product with 2 µl of 6 x loading dye , then, the blend was placed into the well with the using of DNA ladder with a molecular weight 1 K b p Ladder & 100 b p . (Accu Bioneer/Korea) as a molecular size marker via the agarose gel at 90 V. for 1 hr. (Sigma Chemicals Co. USA) (ethidium bromide dye) with a concentration (0.5µg/ ml) [63-64] , then, visualized on a gel documentation system (Biometra, Germany) according to previous study technique [22] .

Statistical Analysis

Data evaluated by Chi – Square (χ 2) test , with P - value of ≤ 0 . 0 5 .

Result & Discussion

The CVD are the supreme public reason of adult mortality in the world. More than 80 % of CVD mortality occurred in low - & middle - income nations & arise almost equally in men & women. The chief reasons of cardiovascular diseases are tobacco use, unhealthy diet, physical inactivity, high blood pressure & high blood cholesterol [24] .

The correlation of coronary heart disease & periodontal disease may be due to fundamental response trait, which seats an individual at high risk for having both of periodontal disease & atherosclerosis [25] .

Atherosclerosis is a chronic disease happening at places of blood movement harassment & is the main reason of severe illness, loss of creative life years & passing away among individuals all over the world. The infectious hypothesis of atherosclerosis has been studied for some decades, simulating participation of both innate & adaptive immune systems in atherogenesis, as well as, human atherosclerosis can be considered both a metabolic & an inflammatory disease [26, 27] .

However, despite of the several investigations were published microbial infections & recommended them as an important contributing feature in pathogenesis of atherosclerosis, the effectiveness of the indications for the greatest of the Periopathogen related with atheromatous plaque of cardiovascular disease is truthful., In major of the cases bacteria surrounded by a biofilm matrix convert to be more resistant to specific antibiotics & the host immunity system than are planktonic forms of the same bacteria, possibly cumulative the existing difficulties connected to medical biofilms [28] & particularly to bacterial biofilms probably related with the pathogenesis of atherosclerosis [29] .

In this study, DNA molecules of *Treponema denticola* was detected in (45/100) 45% of cardiovascular atheromatous plaque samples, this result was in accordance with the previous correlated studies that reported presence of DNA samples of *T. denticola* in (49.01 %) of atherosclerotic plaques & (39.21%) in both sub gingival & coronary atheromatous plaque samples [21] as well as, the proportion of *T. denticola* DNA samples in the current study was greater than the ratios of other corresponding studies that revealed DNA samples of *T. denticola* were (6%) & (23.1%) in atherosclerotic samples [30, 22] respectively, & less than in other related studies which mentioned the ratios of *T. denticola* DNA samples in sub gingival plaques & atheromatous plaque lesions was (54.5%) [31, 21]. Indeed, Mahendra *et al*. reported presence of periodontal bacteria in Coronary Heart Disease patients & *T. denticola* was meaningfully improved from 4 1 . 2 % to 6 6 . 7 % in patients with Coronary Heart Disease [32] .

Furthermore, other researchers ensure these details & published *T. denticola* can infiltrate gingival tissues & entering the blood vessels, with chance to attack the heart & cardiovascular epithelium in middle to great arteries containing aorta, coronary & carotid arteries [33] . Additionally, the role of *T. denticola* in pathogenesis of atheromatous plaque of coronary heart disease achieved via numerous pathways , counting straight arterial contagions, endothelial dysfunction, initiation of the inflammatory response, producing variations in the lipid profile as well as change the expression of specific genes [34] .

Moreover, distribution of demographic data in the current study demonstrated the existence of *T. denticola* in cardiovascular atheromatous plaques was significant in male 28 /45 (62 %) more than 17/45 (38%) in female atherosclerotic patients (P = 0.07) table (3) these findings were compatible with correlated study [35] & these finding may be related to the estrogen hormone in females, as we know estrogen is one of the defense aspects against coronary heart disease in females since estrogen can control numerous metabolic feature such as lipid, inflammation marker, & coagulation system. Estrogen similarly has vasodilatation against α & β receptors in blood vessel walls [36] . Otherwise, these finding may be due to the greater number of the males [63] in comparison to the number of the females [37] in the current study [63-64] .

Furthermore, positive atherosclerotic plaques with *T. denticola* DNA samples were present in elderly patients above 50 years old 23/45(51%) approximate to 22/45(49%) in atherosclerotic vessels patients younger than 50 years old with no significant importance, table (3), these finding may be due to decrease the activity of immune system (adaptive immune response to bacterial infections) in affected participants which was in line with closely related study referred that oldness moderate adaptive immune response , in excess of innate immune response , reduction in immune system will rise susceptibility & occurrence to contagions [30, 37]. These results may unpredictable thru the results of Janket & colleagues [38] , Mattila & colleagues [39] that suggested the periodontal disease might generate a greater risk for CVD between younger contributors.

Besides, there were more male Coronary Heart Disease patients compared to female. These findings were in line with the study done by Mosca *et, al.,* [36] that showed the prevalence of male Coronary heart disease patients is higher for every age groups up to 75 years old.

On the other hand, in the current study detection of *Treponema denticola* in atherosclerosis plaque samples was accomplished up on the species specific *16S rRNA gene* of these spirochetes as demonstrated in figure no. 1, & several corresponding researches have largely relied on Conventional & Real Time PCR technique with *16S rRNA gene* for the detection & quantification of various Periopathogen including *T. denticola* DNA in oral [16, 35, 40] & systemic microbiome including cardiovascular atheromatous plaques (30, 34) & the identification method used in this study proved to be a reliable & sensitive assay [66 -71] .

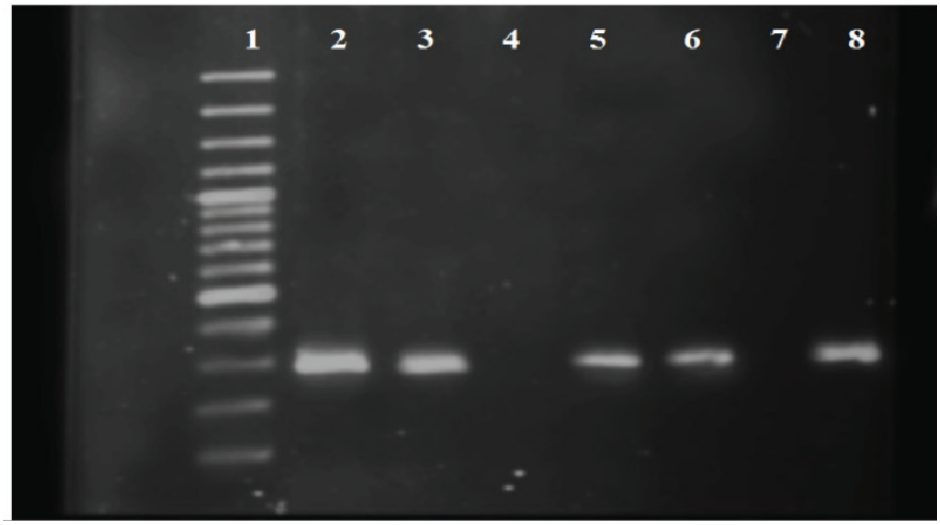

Figure 1. Amplified DNA samples of Athermanous plaque specimens of patients with vascular diseases represented by 316 – b p of *Treponema denticola* 1 6 S r R N A gene segment . Lane 1 DNA Ladder 1 0 0 b p., Lanes (2,3,5,6 & 8) positive specimens , & (4,7) negative samples.

Moreover, in the present study, *T. denticola* DNA molecules were detected in 35/45(78%) of blood vessels atheromatous plaques of (type1 & 2) diabetic patients higher than those (1045/)22% whom non diabetic (p = 0.07) & *T. denticola* did not displayed (2/00) 0 % in control group table (3).

Table 3. Prevalence of *Treponema denticola* In Atherosclerotic Vessels & Diabetic patients

Variable		Atherosclerotic Patients 45 /100 (45%)		Diabetic Patients 35 /45 (78%)		Statistical analysis
		(+) No., Percentage	(-) No., Percentage	(+) No., Percentage	(-) No., Percentage	
Gender	Male	28 /45 (62 %)	35 /55 (64 %)	23 /35 (66%)	5/10 (50 %)	X2= 3.52 DF= 1 P = 0.07
	Female	17/45 (38%)	20 /55 (36 %)	12/35 (34%)	5/10 (50 %)	
	Total	45 / 100	55 / 100	35 / 45	10 / 45	
Age	38-50 years	22/45 (49 %)	17 /55 (23 %)	15 /35 (43%)	3 /10 (30 %)	X2= 0.288 DF= 1 P = 0.59
	> 50 years	23/45 (51 %)	28 /55 (77 %)	20 /35 (57%)	7 /10 (70 %)	
	Total	45 / 100	55 / 100	35 / 45	10 / 45	

These findings (presence the highly putative Periopathogen *T. denticola* in atheromatous plaques of diabetic patients) may submit an evidence for participating of the most Periopathogen invader in polymicrobial biofilms in local periodontitis & chronic systemic infections such as cardiovascular atherosclerosis as well as diabetes mellitus, this phenomenon was in line with various epidemiological surveys have recommended that Periopathogen may be considered as an important risk factor & possibly corelated to the development & development of atherosclerosis & myocardial infarction & periodontal disease can be tightly connected with diabetes & cardiovascular diseases [10, 25, 41, 42].

It is necessarily to demonstrate that one of the virulence factors of *T. denticola* it is ability to in greatly gluey media & it is probable that in polymicrobial biofilms, *T. denticola* intermediates the renovation of biofilm constructions & that the ensuing improved nutrient flow permits a higher biofilm, biomass to be continual. [43] , in biomass Polymicrobial biofilms, *T. denticola* consistently have a synergistic effect in these biofilm formation with *Porphyromonas gingivalis* in periodontal environment & then, this property offers many chances for recurring distribution of these pathogens in the blood circulation more simply & can consequently get occupied in the coronary atherosclerotic plaques [44] . .

Several authors also rigorously reported smoking, type 1,2 diabetes mellitus, hypertension, hyperlipidemia, male sex & advanced age are considered as risk factors for atherosclerosis [45, 46,47]. Indeed, recent studies proposed the periodontal disease as a risk factor to atherosclerotic cardiovascular diseases because of the endothelial damage caused by the toxins of the periodontal pathogens [21, 48, 49,] & this effect (endothelial damage) is related to the host immune response (inflammatory & adaptive immune response) contribute to the progress of these conditions [10, 50].

In spite of few studies stated distinct role of periodontal disease in sub-clinical atherosclerosis [51] , the percentage of oral bacteria in atherosclerotic plaques was extraordinarily low, as well, the microbiome pattern of atherosclerotic plaques was totally diverse from that existing in periodontal microbiome, that revenue, oral pathogens did not straightly encourage the atheromatous plaque formation. Thus, the metabolic yields of the oral microbiome, or the host's inflammatory & immune response, might indirectly affect the atheromatous plaque configuration [52] .

Moreover, in 2012, according to the American Academy of Periodontology which demonstrated that the current studies have not yet offer any provision to the underlying association among the cardiovascular & periodontal disease. Some studies also confirmed incompatible consequences.[53]. Whereas, after an extensive review of the literatures, the American Heart Association confirmed that periodontitis & Periopathogen were independently associated with arteriosclerotic vascular diseases, these relationships were demonstrated with level A evidence, they proposed that there were numerous reasonable mechanisms by which Periopathogen could be associated with arterial disease [54, 72] .

In overall, many chronic diseases like cardiovascular atherosclerosis & diabetes mellitus can be caused from person's risk factors, non - modifiable risk factors like family history, increasing age, male gender, menopause, & race activities are risk factors which cannot be changed, while changeable risk factors can be modified, controlled, or treated. The more risk factors of our population such as Periopathogen have the larger coincidental for our families for evolving cardiovascular diseases as enhanced in comparable studies [55].

In fact, about [55] corresponding studies according to periodontal disease, atherosclerosis & *Treponema denticola* were identified, *in vitro* & *in vivo* studies were published between 2002 & 2020 on PubMed. & elevating related insights about the role of *T. denticola* & the related Periopathogen *P. gingivalis* & their association with the systemic diseases such as atherosclerosis, most of them focused on the bacterial pathogenesis for tissue invasion & development of polymicrobial biofilms & existence of *T. denticola* with its putative risk factors like dentil sin activity & pathogenicity that involved in proteolysis function [56], other risk factors were reported to be involved in cell invasion, bacterial co-aggregation, nutrient uptake, complement activation, evasion of the host immune response, inhibition of the hemostasis system [49, 52].

Furthermore, the highly existence of Periopathogen within the blood circulation & the synergistic effects with other oral pathogens in cardiovascular tissues, these evidences can interpret the strong associations with atherosclerosis, so, they can conclude that the invasion of highly resistant, putative periodontal pathogens & their toxins associated to the host's immune mechanism & inflammatory response may contribute to the development & progression of cardiovascular atheromatous plaques & atherosclerosis, with diabetes mellitus as well improved in various recent correlated studies [10, 57, 58, 70] .

Conclusion & Recommendation

Our study revealed the presence of DNA molecules of putative periodontal pathogen *Treponema denticola* in cardiovascular & diabetic atheromatous plaques. these findings represent a clear evidence that periodontal pathogens can arrive to the blood circulation, producing transient bacteremia , then, can aggregate in unhealthy blood vessel walls which propose the extra conceivable risk factor that intermediate with pathogenesis of periodontal bacterial infection & genesis of atherosclerosis

& may be associated with diabetes. These annotations can offer visions into the pathogenesis of cardiovascular atherosclerosis & diabetes with periodontitis & help to plan protective behavior plans counting primary oral prophylaxis for this life threatening disease.

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