Anaerobe 44 (2017) 66–72

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Anaerobes in human infections (including dental/oral infections)

"Association of periodontopathic anaerobic bacterial co-occurrence to atherosclerosis" – A cross-sectional study

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ARTICLE INFO

Article history:
Received 7 July 2016
Received in revised form
14 January 2017
Accepted 4 February 2017
Available online 8 February 2017

Handling editor: Kaori Tanaka

Keywords:
Anaerobic bacteria
Atherosclerosis
PCR
Periodontitis
Virulence genes

ABSTRACT

Background: Epidemiological studies have shown a link between periodontitis and atherosclerosis. Hence the present study was chosen to assess the presence of eight anaerobic periodontal pathogens and their virulence genes in subgingival plaque (SGP) and atheromatous plaque (AP) of patients with Ischaemic heart disease.

Methods: SGP and AP collected from 65 Ischaemic heart disease patients were screened for the presence of periodontal bacterial pathogens by Polymerase chain reaction. The samples positive for *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* were screened for virulence genes. Chronic periodontitis patients (ChP) without any systemic disease (n = 59) and healthy subjects free of both periodontitis and systemic disease were included as control groups (n = 100).

Results and discussion: Statistical significance was observed for the prevalence of *16S rRNA* of *P. gingivalis, T. forsythia, T. denticola* and *P. nigrescens* both in SGP and AP. Nine different periodontal bacterial co-occurrences were observed in SGP and AP of Ischaemic heart disease patients. Besides, the prevalence of these nine different bacterial co-occurrence was high in SGP OF Ischaemic heart disease patients compared to ChP without systemic disease. Among the nine different bacterial co-occurrence, only four were observed in SGP of ChP without systemic disease in spite of high prevalence of these anaerobic bacterial species. While, bacterial co-occurrences was completely absent among healthy subjects. Significant odds and risk ratio to atherosclerosis were observed for *P. gingivalis*, *T. forsythia*, *T. denticola* and *P. nigrescens*. Among the virulence genes, significance to atherosclerosis was observed for *P. gingivalis* type II *fimA* and *T. forsythia bspA*.

Conclusion: The results of this study strongly correlate periodontal bacterial co-occurrence and periodontal bacterial adhesion factor to atherosclerosis.

1. Introduction

Inflammation plays a significant role in the pathogenesis of atherosclerosis. Earlier studies have suggested chronic infectious diseases as a risk factor for atherosclerosis and acute cardiac syndromes. More than a decade, epidemiological studies have frequently shown a link between coronary heart disease and periodontal disease. While a direct link has not yet been confirmed, periodontitis is associated with an increased risk of myocardial infarction and stroke [1]. Periodontitis involves infection induced by diverse anaerobic Gram-negative bacteria that invade superficial and deeper gingival tissues [2]. Chronic periodontitis, a persistent bacterial infection is characterized by several incidents of bacteremia resulting in the migration and accretion of periodontal pathogens on the coronary plaques [3]. Severe chronic generalized periodontitis results in systemic inflammation and endothelial dysfunction. The dental plaque associated anaerobic periodontal bacteria are capable of invading into coronary vessel [4,5]. It is well recognized that anaerobic Gram negative bacteria (*Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Campylobacter rectus, Eikenella corrodens, Aggregatibacter actinomycetemcomitans, Prevotella intermedia* and *Prevotella nigrescens.*) in emergent dental plaque is likely to overpopulate saccharolytic Gram-positive

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http://dx.doi.org/10.1016/j.anaerobe.2017.02.003
1075-9964/

bacteria [6].

The fimbriae and collagenase virulence factors of *P. gingivalis* could contribute to the initiation of chronic periodontitis. *P. gingivalis* may colonize to gingival crevices by the fimbriae-mediated adherence to gingival epithelial cells [7]. The adhesive capacity of *P. gingivalis* is mainly mediated by the fimbriae, while collagenase and proteases may play an adjunctive role. Among the six fimbrial types of *P. gingivalis,* type II *fim*A is significantly associated with clinical conditions of periodontitis [8]. Collagenase encoded by the *prtC* gene is perhaps the most important of the *P. gingivalis* proteolytic enzymes. If expressed *in vivo,* it is a major destructive enzyme (virulence factor) associated with the soft tissue destruction characteristic of human periodontitis [9,10].

In *T. forsythia* BspA (*Bacteroides* surface protein A), a surface, as well as secreted protein, has been shown to trigger the release of bone-resorbing pro-inflammatory cytokines from monocytes [11] and chemokine IL-8 from gingival epithelial cells by activating the TLR-2-dependent pathway [12]. Besides, BspA has been shown to mediate bacterial adherence and invasion into epithelial cells [13]. A direct evidence for an *in vivo* role of BspA in pathogenesis was reported by a study which demonstrated that a BspA-defective mutant was significantly less potent than the wild-type strain in inducing the alveolar bone loss in mice [14]. The BspA protein, encoded by *bspA* gene of *T. forsythia* thus represents an important virulence factor for *T. forsythia* with multifunctional activities involved in bacterial pathogenesis [15]. Cross-sectional clinical studies have demonstrated an increased risk for periodontitis leading to periodontal attachment loss in the presence of high levels of *T. forsythia prtH* genotype [16,17]. In addition this cysteine protease, encoded by *prtH* gene may play a role in the changeover of a commensal organism to an opportunistic pathogen. Factor H-like binding protein-1 (FhbB), a small surface-exposed lipoprotein has been identified in *T. denticola* which facilitates adherence to factor H-like protein −1 present on anchorage-dependent cells and in the extracellular matrix. In addition factor H-like binding protein-1 leads to C3b inactivation [18].

To determine the incidence of anaerobic periodontopathic bacterial co-occurrences in periodontitis and atherosclerosis, the presence of *16S rRNA* of periodontal pathogens *viz P. gingivalis, T. denticola, T. forsythia, C. rectus, E. corrodens, A. actinomycetemcomitans, P. intermedia* and *P. nigrescens* were screened in the atheromatous plaque (AP) and subgingival plaque (SGP) samples obtained from the same patients with Ischaemic heart disease. In addition, genes that code for significant virulence factors among the highly prevalent bacterial species (*P. gingivalis (fimA type II, prtC), T. forsythia (bspA, prtH) and T. denticola* (*fhBb*), were detected in AP and SGP samples of Ischaemic heart disease patients and SGP of control groups. Chronic periodontitis (ChP) patients with absence of systemic disease and healthy subjects free of both periodontitis and systemic disease were included as control groups.

2. Materials and method

2.1. Study population

Sixty-five atherosclerosis patients with Ischaemic heart disease procedure in a tertiary care hospital in Chennai were enrolled for the present study. The control group included ChP (n = 59) patients without systemic diseases and healthy subjects free of both periodontitis and systemic disease (n = 100). The study was cleared by the institutional ethics committee, Dr. A.L.M Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai. Informed consent was obtained from Patients fulfilling the following inclusion criteria.

Inclusion criteria for Ischaemic heart disease patients were patients with coronary artery disease, age between 40 and 72 yrs, a minimal presence of 14 natural teeth and no history of systemic illness or any other oral disease. Edentulous patients, critically ill cardiac patients, patients with diabetes, patients with a history of smoking and pan or tobacco chewing were excluded.

The inclusion criteria for the control group were age between 40 and 65 years, more than 3 teeth with pocket probing depths exceeding 4 mm, bleeding on probing. Patients on periodontal therapy, dental hygiene procedure in the last year, pregnant women and patients with systemic infections were excluded.

2.2. Specimen collection

After careful removal of supragingival plaque, SGP from three different sites was collected from ChP patients and Ischaemic heart disease patients using sterile Gracy curette. Samples from three different sites were pooled in 500 μl of phosphate-buffered saline (PBS, pH 8.0) and stored at −20 °C until assay.

Vascular tissues of the Ischaemic heart disease patients were collected in phosphate buffered saline from the Department of Pathology, Frontier Lifeline (Dr.K.M.Cherian Heart Foundation) Chennai. Vascular tissues were stored at −20 °C until assay.

2.3. DNA extraction

SGP samples in phosphate buffered saline were centrifuged, the deposit resuspended in 150 μl of lysis buffer (10 mM/L Tris-HCl, 1.0 mM/L EDTA, 1.0% Triton X-100, pH 8.0) and boiled for 10 min. Ten μl of the supernatant was used as the template for PCR. AP was removed with the help of sterile blade from vascular tissue and placed in 150 μl of lysis buffer and the genomic DNA was extracted by boiling - lysis method [19].

2.4. PCR assay

Detection of *16S rRNA* gene of eight anaerobic periodontal pathogens was performed as per the protocol of Ashimoto et al., 1996 [20]. Primers used for the study are shown in Table 1.

PCR amplification was performed in a volume of 50 μl containing 5 μl of the template, 5 μl of 10X PCR buffer and 1 U *Taq* polymerase, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, and 0.8 μM primer for detection of *P. gingivalis fimA* type II gene. The PCR temperature profile was followed as per Amano et al., 1999a [21].

For detection of *P. gingivalis prtC* gene, PCR amplification was performed in a volume of 50 μl containing 5 μl of the template, 5 μl of 10X PCR buffer and 1 U *Taq* polymerase (Invitrogen), 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, and 25 pM primers. PCR thermocycling condition for *prtC* gene included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C/1min, annealing at 50 °C/min, extension at 72 °C/1.5 min and subsequently, a final extension step at 72 °C for 7 min.

PCR amplification was performed in a volume of 25 μl containing 5 μl of the template, 2.5 μl of 10X PCR buffer and 1 U *Taq* polymerase, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, and 25 pM primers for detection *of T. forsythia bspA, T. forsythia prtH,* and *T. denticola fhbB* gene. PCR thermocycling condition included an initial denaturation at 94 °C for 2 min, followed by 36 cycles of denaturation at 95 °C/30 s, annealing at 60 °C/1min, extension at 72 °C/1 min and subsequently, a final extension step at 72 °C for 2 min. PCR reactions were performed in duplicate.