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Research Article

PREVALENCE OF *PROPHYROMONAS GINGIVAL* IS AMONG CIGARETTE SMOKERS AND NON-SMOKERS CHRONIC PERIODONTITIS SUDANESE PATIENTS

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ABSTRACT

Background: *Porphyromonas gingivalis* is a major periopathogen that involved in the pathogenesis of chronic periodontitis. Cigarette smoking is a major risk factor in the development and further progression of periodontal diseases.
Methods: 163newly diagnosed chronic periodontitis patients were recruited randomly from patients attending Khartoum Teaching Dental Hospital according to criteria (pocket depth ≥ 5 & bleeding on probing). 89 were non-smokers while 74 were smokers. The two groups were examined clinically for indicators of periodontal health status. Plaque Index, Gingival Index [Probable Pocket Depth, Gingival Recession, Clinical Attachment Loss, Furcation Involvement and Tooth Mobility scores. The subgingival plaque samples were obtained from each subject, DNA extraction of *P.gingivalis* and quantification with SYBR Green I assay real-time PCR technology was done.
Results: Different means of periodontal health indicators showed highly significant differences between smokers and non-smokers using t-test ($P < 0.001$). In contrast to non-smokers, smokers tend to have more plaque accumulation, less gingival bleeding, deep probable pockets, severe gingival recession, advanced clinical attachment loss, worst furcation involvement and more tooth mobility. The mean log-transformed of absolute counts of *P. gingivalis* in subgingival plaque samples from the smokers was 212.93 ±44.95CN/1000 copies while the figure for non-smokers was 37.30 ±5.24CN/1000 copies using Independent t-test ($P = 0.001$).
Conclusion: Smokers showed more plaque accumulation, gingival recession, pocket depth, clinical attachment loss, furcation involvement tooth mobility but less gingival bleeding than non-smokers. Tobacco smoking modifies subgingival microbiota particularly *P.gingivalis* in chronic periodontitis patients.

INTRODUCTION

Porphyromonas gingivalis, Tannerella forsythensis, and *Aggregatobacillus actinomycetemcomitans* are considered true& majorperiodontal pathogens [1]. *Porphyromonas gingivalis* belongs to the genera Porphyromonas from the family *Bacteroidaceae*. It is a gram-negative oral anaerobe that clearly fulfills Socransky etiologic criteria (1994) [2]. It is one of the terminal "Red complex" organism and late colonizers. It is involved in the pathogenesis of chronic periodontitis [3]. It can successfully colonize, invade periodontal tissues and evade the host defense mechanism that results in host tissue damage. It has many virulence factors as proteolytic enzymes that cause collagen degradation as well as other exotoxins and gingipains which make many proteins susceptible to further cleavage. *P. gingivalis* has capsule and fimbriae by which it can attached to epithelium, connective tissues and other bacteria.

Periodontal disease is a widespread condition ranging from simple gingival inflammation to severe periodontal breakdown [4]. Chronic periodontitis is a multifactorial disorder. Microbial dental plaque biofilms are the principal etiological factor of periodontitis. Oral cavity has a complex and diverse microbial flora comprising more than 500 different bacterial types [5&6]. Diabetes mellitus [7] (especially in individuals in whom metabolic control is poor) & tobacco smoking is the major systemic risk factors for periodontal diseases [8&9].

Periodontal health is likely to be adversely affected by smoking. Tobacco smoking inhibits gingival inflammation [10] that is mainly attributed to gingival vasoconstriction [11] &

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compromising the immune response [12]. It can exert deleterious effects on PMN and other neutrophil functions as chemotaxis & phagocytosis [13&14]. Tobacco smoking modify the production of pro-inflammatory cytokines interleukin-1 [15] & TNF-alpha [16]. Tobacco nicotine can exert cytotoxic effects on periodontal fibroblast functions [17] & inhibit the production of fibroblast fibronectin & collagen; stimulate fibroblast collagenase activity [18]. In addition tobacco smoking can stimulate osteoblasts alkaline activity [19].

In Sudan, studies investigating the possible effects of smokeless tobacco (toomback, also called saffa) on periodontal health are scarce. These studies revealed that the high prevalence of oral cancer in Sudan is largely due to the chronic use of toomback [20]. The association between Saffaand development of oral carcinoma is likely to be causal [21]. Smokeless toomback is one of the major risk factor for oral cancer in Sudan [22].

The imbalance of the host-bacterial interactions in smokers that result in increasing the prevalence and severity of periodontal destruction may be due to change in the composition of the subgingival plaque. Few studies have examined the oral microbial flora of smokers and non-smokers. Study by Haffajee & Socransky (2001) [23] in USA, reported on, the relationship of cigarette smoking and the subgingival microbiota. The authors reported that members of the orange and red complexes were significantly more prevalent in current smokers than in non-smokers and former smokers. On contrary another study by Apatzidou *et al*. (2005) [8] in England, reported on, the impact of smoking on the clinical, microbiological and immunological parameters of adult patients with periodontitis. The study indicated that, the subgingival microflora of smokers appears similar to that of non-smokers. Due to the controversial results and different outcomes further studies are needed to define the effects of tobacco use on bacteriological and tissue destruction in periodontitis.

Since smoking is a habit which is practice in every society including the Sudanese, periodontal health is likely to be adversely affected by that unhealthy habit. This study was designed to detect and quantify *P. gingivalis* using Real-time PCR in subgingival samples of chronic periodontitis patients & to investigatecommon manifestations ofperiodontal disease among Sudanese smokers and non-smokers.

MATERIALS AND METHODS

The study received ethical clearance from the research committee -faculty of dentistry - university of Khartoum. Written informed consents were obtained from all studied subjects. All studied patients with periodontal problems received standard management.

The study involved163 newly diagnosed patients with chronic periodontitis. The studied patients were recruited from out-patient clinics of Khartoum teaching dental hospital (KTDH) – Khartoum - Sudan. Only patients with anage ≥ 40 years, clinical attachment loss > 3 mm, did not used antibiotics in the past three months, cigarette smokers and non- smokers were included. Patients with aggressive periodontitis, using alcohol, snuff & or shisha, usingoral contraceptive or pregnant and those suffering from a systemic disease that modifies or aggravates periodontal diseases (e.g. diabetes mellitus, blood dyscrasias & HIV) were excluded.

The sample was (163). The smoking status of the subjects was assessed by self reported questionnaire. Smoking exposure was expressed in terms of the number of cigarettes consumed per day and the duration since the start of cigarette smoking. Proper clinical examination was done for each patient to assess periodontal health indicators including plaque index (PI)[24], gingival index (GI)[25], probable pocket depth (PPD)[26], gingival recession (R)[27], Clinical attachment loss (CAL)[26], furcation involvement[28]& tooth mobility[29]. Sextants were used according to Federation Dental International (WHO 1983) [30].

For subgingival Plaque sampling and microbiological documentation, each subject of chronic periodontitis patients the mesial surface of molars of the selected posterior sextant (pocket depth ≥ 5 & bleeding on probing) were sampled using sterile paper points (size - 40) for 20 seconds after removal of supragingival plaque the outcome was pooled in 1.5 ml of reduced transport fluid. The samples were stored at -20˚C until DNA extraction. Nucleic acid extraction kit was used (Vivantis-GF-1) (tech@vivantis.com) for high quality tissue genomic DNA isolation. For the detection of *P*. gingivalis in the DNA samples an extremely powerful molecular tool, real-time PCR (polymerizing chain reaction) amplification of the 16S small- subunit rRNA gene was used [31]. SYBR Green I assay real-time PCR technology was performed with primers and an interchelating dye specific for the *P. gingivalis* 16S rRNA gene. The sequences of primers of *P.gingivalis* were:

F-primer: 5'-GCGCTCAACGTTCAGCC-3' (17mer) Product size (100pmol/ul) F (1,001)
R- primer: 5-CACGAATTCCGCCTGC-3 (16 mer) Product size (100pmol/ul) R (894)

thatobtained as pre-validated, optimized and ready to use from OligoTM MACROGEN (World Meridian 10F, Gasan-dong, Geumcheon-gu, Seoul, 153-781 KOREA). In addition to primers, an interchelating dye (RealMOD Green) Real –time PCR master mix Kit (iNtRON Biotechnology WWW.intronbio.com) was used. Also a positive control (QUB Lab Belfast BT 12 6BP) as quantification standard was used in a 10-fold serial dilution, to construct standard curves for absolute quantification of the test species, and to confirm efficiency, linearity and sensitivity of the assays. Due to extreme sensitivity of PCR, bacterial threshold levels above which a positive PCR result was of clinical importance, were determined. A single closed tube for both PCR and the detection of amplified products were used to avoid DNA contamination problem.

Specificity of primers was initially confirmed by blasting them against a positive control sequence (10-fold serial dilution) from (QUB Lab Belfast BT 12 6BP) followed by disassociation curve analysis [Figure 1]. A single disassociation peak of the positive standard was used as a proof of specificity of the primer set [Figure 2]. To further confirm specificity, primers set was tested against a pooled subgingival DNA sample of chronic periodontitis patients using a SYBR Green real-time PCR assay followed by curves analysis [Figure 3&4]. At each cycle accumulation of PCR products was detected by

monitoring the increase in fluorescence of the reporter dye from the dsDNA-binding SYBR Green. After the PCR, amplification and standard curves were constructed in a range of 60˚ C to 95˚ C. All data were analyzed using the Rotor-Gene 6000 series software (QIAGEN).

Quantitative PCR assays were performed in 20µl reactions, consisting of 10µl master mix, 2 µl F-primer, 2 µl R-primer, 0.5 µl HRM dye, 5 µl template DNA (or positive standard) and 0.5 µl PCR-grade water on a QIAGEN (Rotor-Gene 6000 series USA) real-time PCR system using the following thermocycling program: an initial amplification cycle of 50˚ C for 2 min and95˚C for 10 min, followed by 45 cycles at 95˚ C for 15 sec and 60˚C for 1 min.

Statistical evaluation was performed using the Microsoft Office Excel (Microsoft Office Excel for windows; 2007) and SPSS (SPSS for windows version 11.5for clinical finding & 19 for microbiological ones). Comparisons between the two groups according to plaque Index, gingival Index, probable pocket depth, clinical attachment loss, gingival recession, furcation involvement, tooth mobility & bacterial load were done using appropriate statistical tests. P < 0.05 was considered significant.

RESULTS

45.4% of the patients with chronic periodontitis (N =163) were cigarette smokers. All smokers (N =74) were males while (70.8%) of the non-smokers (N = 63) were females. There is significant association between cigarette smoking and male gender (P < 0.001).

Different means of periodontal health indicators showed highly significant differences between smokers and non- smokers using t-test (P < 0.001) [Table 1]. In contrast to non-smokers, smokers tend to have more plaque accumulation, less gingival bleeding, deep pockets, severe recessions, advanced clinical attachment loss, worst furcation involvement and more tooth mobility.

Microbiological findings showed that *P.gingivalis* was detected in 100% of the samples. The Quantitative PCR assays for *P.*gingivalis showed excellent linearity (R^2>0.99) over a dynamic range of 10^{-3}-$10^{0.5}$ ng/µl concentration for reaction [Figure 4]. In SYBR Green- immune assay, primer set produced single dissociation peak indicating high specificity [Figure 2]. PCR inhibition wasn't encountered as assessed by the total bacterial assays [Figure 3]. Threshold cycle values obtained were converted into absolute counts and copies per reaction using the standard curves; values were then converted into copies per sample by multiplying by a factor of 20(since 5 µl of the eluted DNA was included in the reaction). The mean log-transformed of absolute counts of *P. gingivalis* in sub gingival plaque samples from the smokers was 212.93 ±44.95 CN/1000 copies while the figure for non-smokers was 37.30 ±5.24 CN/1000 copies using Independent t-test (P = 0.001) [Table 1]. The comparison of difference in mean scores was statistically highly significant using independent t -test (P=0.001).

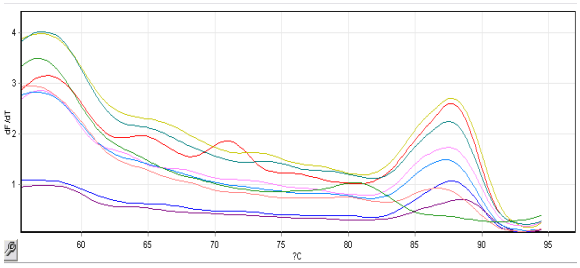

Figure1 Dissociation curve of real time PCR for *Prophyromonas gingivalis* constructed according to SYBERGREEN assay.

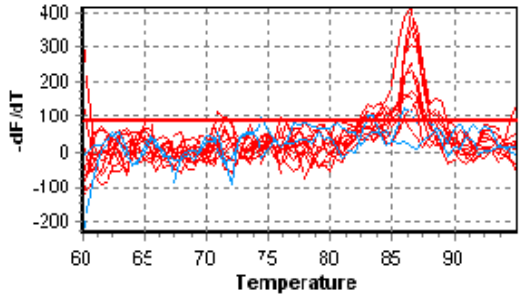

Figure 2 Melting curve analysis of amplification products of primer set result in only one peak of real time PCR for *Prophyromonas gingivalis* constructed according to SYBERGREEN assay.

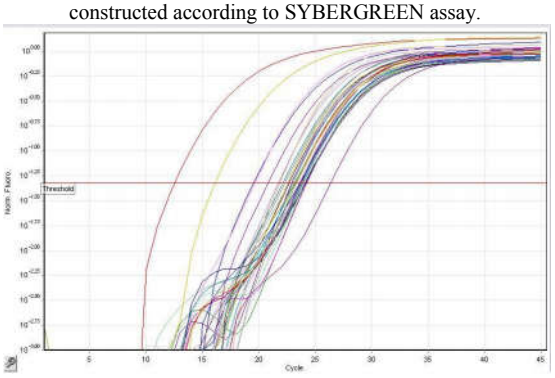

Figure 3 Amplification curve of real time PCR for *Prophyromonas gingivalis* constructed according to SYBERGREEN assay.
(The horizontal bar indicates the threshold line the horizontal axis shows the threshold cycles while the vertical axis shows the fluorescence).

Table 1 Means of clinical findings according to smoking status in chronic periodontitis patients (n = 163)

Parameter	*Mean±SE N=163*	*Non-smokers Mean±SE N=89*	*Smokers Mean ±SE N=74*	P. value
Plaque Index	2.65± 0.04	2.39 ± 0.49	3.00±0.00	<0.01*
Gingival Index	1.55±0.04	1.96±0.02	1.00±0.00	<0.01*
P. Pocket Depth (mm)	2.21±0.08	1.54±0.06	3.10±0.1	<0.01*
G. Recession (mm)	1.82±0.13	0.57±0.09	3.49±0.06	<0.01*
PAL (mm)	4.36±0.23	1.97±0.13	7.53±0.07	<0.01*
Furcation involvement	1.50±0.05	1.04±0.03	2.11±0.04	<0.01*
Tooth Mobility	1.43±0.05	0.97±0.04	2.04±0.04	<0.01*
Bacterial load (CN/1000) copies	117.03±45.23	37.30 ± 5.24	212.93 ± 44.95	<0.01*

*Statistically significant

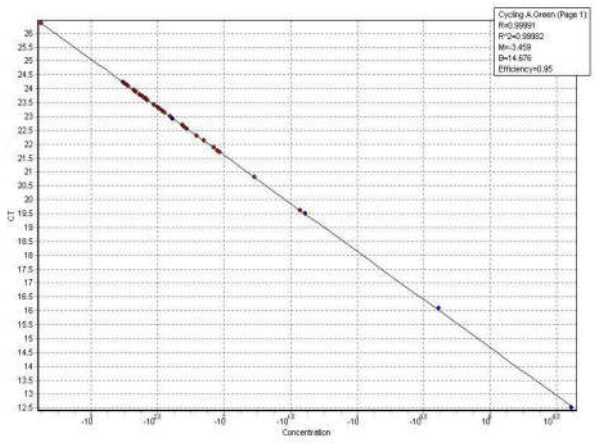

Figure 4 Standard curve of real time PCR for *Prophyromonas gingivalis* constructed according to SYBERGREEN assay.
(The horizontal axis indicates the concentration while the vertical axis shows the threshold cycles (CT): correlation coefficient = 0.99: Data analyzed by Rotor-Gene 6000 series software).

DISCUSSION

In the present study smoking was found to be more prevalent among males than females and that may be due to social impact in Sudan. Most of periodontal indicators were consistent to previous studies. Greater pocket depth [38], more gingival recession[39], more clinical attachment loss[39 & 40], more furcation involvement [41 & 42], more tooth mobility [43 & 44] and less gingival bleeding [36 & 37] in smokers were similar to previous studies. However plaque accumulation was significantly higher in smokers favoring the controversy towards Bergstorm (1981) [32] and Macgregor *et al*. (1985) [33] against Bergstrom and Eliasson (1987) [34] and Bergstrom (1989) [35] [Table 1].

The result of Quantitative PCR assays for *P.gingivalis* showed that *P.gingivalis* was detected in 100% of the samples of the participants. No PCR inhibition was encountered as assessed by the total bacterial assays [Figure 3]. The results showed excellent linearity (R^2>0.99) over a dynamic range of 10^{-3}-$10^{0.5}$ ng/µl concentration for reaction (Figure 4). In SYBR Green-immune assay, primer set produced single disassociation peak indicating high specificity [Figure 2]. Smokers showed more absolute counts of *P. gingivalis* than non-smokers, mean 212.93 ± 44.95CN/1000 versus 37.30 ± 5.24CN/1000 [Table 1]. The comparison of different means for absolute count of *P. gingivalis* among smokers and non-smokers was statistically highly significant using independent t- test (P= 0.001). These results were in agreement with those of Zambon *et al*. (1996) [45], Eggert *et al*. (2001) [46], Kamma *et al*. (1999) [47], Umeda *et al*. (1998) [48] and Haffajee and Socransky (2001) [23]. The previous studies suggested that the inferior periodontal health in smokers has been correlated to the presence of greater numbers of specific periodontal microorganisms in smokers than in non-smokers.

CONCLUSION

- Smokers showed more plaque accumulation, less gingival bleeding, more gingival recession, more pocket depth, more clinical attachment loss, more furcation involvement and more tooth mobility than non-smokers.
- Tobacco smoking modifies subgingivalmicrobiota particularly *P.gingivalis* in chronic periodontitis patients.

Recommendations

There is an urgent need to start an intensive education program to the public on the negative health consequences of cigarette smoking. Smoking cessation counseling should be an integral part of any dental and periodontal therapy and prevention program.

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Limitations

In the present study SYBR Green I assay real-time PCR technology was used, which is considered as efficient method; moreover it does not require bacterial viability. Limitation of this method is that the SYBR Green dye is binding to dsDNA which may result in overestimation. 10-fold serial dilution of control positive was used as quantification standard and to construct standard curves to confirm efficiency, linearity and sensitivity of the assays.

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