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Research Article

CLINICAL, MICROBIOLOGICAL AND MOLECULAR STUDY OF *PORPHYROMONAS GINGIVALIS* IN PATIENTS WITH CHRONIC PERIODONTITIS

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ABSTRACT
Aim of the present study was to correlate the clinical condition and concentration of *Porphyromonas gingivalis* in chronic periodontitis patients among smokers and comparing with non-smokers by traditional and newer methods of bacterial identification. Method: 100 patients (50 never-smokers and 50 smokers) in the age group of 33 to 59 years were selected. Gingival index (Loe and Silness) and Bleeding on probing, (sulcus bleeding index), Probing pocket depth, Clinical Attachment Loss were measured. Subgingival plaque was collected; inoculated and molecular detection of *Porphyromonas gingivalis* was done using PCR. Statistical analysis was done using Descriptive statistics, Independent sample t test, Contingency co-efficient test and one way Anova test using SPSS 14 software. Smokers had statistically significant lesser gingival scores and bleeding on probing compared to non-smokers. No statistically significant difference in the P.gingivalis bacterial counts among the smokers and non smokers. A positive co relation was found between the increasing probing pocket depth and clinical attachment loss with the *P. gingivalis* counts. Present study combines both the microbiological and molecular technique to assure a more accurate and rapid method of detection and found that there is no statistically significant difference in the *Porphyromonas gingivalis* load in the subgingival plaque among smokers and non smokers.

INTRODUCTION
Periodontitis is characterized by local infection and inflammation of tooth-supporting tissues, leading to various degrees of periodontal attachment loss in affected teeth. Different forms of periodontitis are multifactorial diseases where microorganisms present in dental biofilms are involved. Etiologic bacteria of periodontal diseases typically include gram-negative anaerobic bacteria; among those, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*), and *Treponema denticola* are strictly anaerobic and *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* and *Campylobacter rectus* are facultative/microaerobic. Several demographic and behavioural characteristics, such as race, age, gender, and smoking, as well as socioeconomic status, appear to be related to the prevalence of periodontitis (Konnen *et al.,* 2007). Smoking is a known risk factor for destructive periodontal diseases, and evidence exists of its role in the pathogenesis of periodontitis (Hujoel *et al.,* 2003). Clinical and laboratory studies have shown an association between decreased immune response and decreased fibroblast activity in smokers and tobacco product addicts. Clinically, smoking has been associated with a reduced inflammatory response of the marginal gingiva, increased probing depth (PD), and greater attachment loss (Haffajee and Socransky, 2001). Microbiota composition could explain, at least in part, the differences between smokers and non-smokers. Although some authors have shown differences in the microbiota between smokers and non-smokers (Shiloah *et al.,* 2000; Haffajee *et al.,* 2001 and VanWinkelhoff *et al.,* 20001) several other studies have not been able to demonstrate relevant differences (Salvi *et al.,* 2005; Natto *et al.,* 2005 and Buduneli *et al.,* 2005). Among the possible explanations for these conflicting findings are inherent limitations of some of the microbiological methods which were previously used (Sabrina *et al.,* 2006). The aim of the present study was to correlate the clinical condition and concentration of *Porphyromonas gingivalis* in chronic

periodontitis patients among smokers and comparing with non-smokers by traditional and newer methods of bacterial identification.

MATERIALS AND METHODS

Study Design and Sample

This cross-sectional study was performed with a convenience sample of consecutive patients seeking treatment (from November 2010 to July 2011) at the Department of Periodontology. 100 patients (50 never-smokers and 50 smokers) in the age group of 33 to 59 years were selected. Smoker is defined as a patient with more than 15 pack years. The participants were eligible for the study if they did not have presence of diseases/conditions that might pose health risks to the participant or examiner or interfere with the results of the study. Specifically, subjects were excluded if they were diagnosed with systemic conditions such as diabetes and cardiovascular disease, had previous periodontal treatment, had used anti-inflammatory or antimicrobial therapy (previous 3 and 6 months, respectively), were pregnant, or were under hormone therapy. Eligible patients willing to participate signed an informed consent. Potential patients were clinically screened and included in the study if they had 12 teeth present (excluding third molars, condemned or endo periodontal lesion-involved teeth). Three of the teeth needed to have at least one site with PD ranging between 6 and 10 mm, and another four needed to have at least one site with a PD of 6 to 10 mm, with visible plaque, marginal bleeding, bleeding on probing (BOP), and clinical attachment loss (CAL).

Clinical Procedures

A full-mouth periodontal examination was performed by a trained and calibrated examiner. Six sites in each tooth were examined (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto lingual). The presence of supragingival biofilm and marginal gingival bleeding were recorded with the Gingival index (Loe & Silness) and Bleeding on probing, (sulcus bleeding index), PD, CAL, and BOP measured with a University of North Carolina probe (UNC-15) manual periodontal probe color coded at 1 to 15 mm. After this subgingival plaque was collected using sterilized Gracey curette by inserting it subgingivally into the deepest portion of the periodontal pocket from the Mesiobuccal surface of the first right maxillary molar, transported using Sodium Thioglycolate medium for microbiological analysis.

Microbiologic Procedure

The subgingival plaque was further processed in the lab as follows.

From the thioglycolate broth, sub cultures were made on blood agar and incubated aerobically as well as anaerobically. Prereduced Sheep blood agar plates were used for anaerobic culture. Simultaneously, a Gram stain smear study was done from the Thioglycolate broth to know the microbiota. The inoculated plates were incubated for 72 hours, and examined for the growth of aerobic and anaerobic bacteria. The black pigmented colonies in the anaerobic plate was confirmed as P.gingivalis by smear examination, biochemically by detection of 16s RNA by PCR. Colony counting was done using colony counter.

Score of 0 = <25,000 CFU/µl
Score of 1+ = 25,000-50,000 CFU/µl
Score of 2+ = 50,000-75,000 CFU/µl
Score of 3+ = 75,000-100,000 CFU/µl
Score of 4+ = >100,000 CFU/µl

PCR Procedure

DNA Extraction was done using Qiagen DNA extraction kit. *P. gingivalis*-specific primers were used to amplify a 404-bp fragment of the 16S rRNA gene.

- Forward primer: 5`AGGCAGCTTGCCATACTGCG 3`
- Reverse primer: 5` ACTGTTAGCAACTACCGATGT 3`

Amplification Reaction Mixtur

Total volume of 20 µl consisting of

- 0.2 mM deoxynucleoside triphosphate mix
- 1.5 mM $MgCl_2$,
- 10X Taq buffer
- 1 µM each primer,
- 1.5 U of Taq polymerase
- 5 µl of template

PCR amplification was performed in a thermalcycler (Eppendorf Pro).

Cycling Parameters

- Initial denaturation at 95°C for 1 min - 40 cycles
- Denaturation: 95°C for 30 s,
- Annealing: 65°C for 1 min,
- Extension: 72°C for 1 min;
- Final extension at 72°C for 2 min.

Statistical Analysis

Was done using Descriptive statistics, Independent sample t test, Contingency co-efficient test and One way Anova test using SPSS 14 software. Statistical significance was accepted at $P<0.05$.

RESULTS

Plaque scores: There was no statistically significant difference in the plaque scores between smokers and non-smokers. (Table 1)

Gingival status: Smokers had statistically significant lesser gingival scores compared to non-smokers. (Table 1)

Bleeding on probing: Smokers had statistically significant lesser bleeding on probing compared to non-smokers. (Table 1)

Probing pocket Depth: There was no statistically significant difference in the pocket depths between smokers and non-smokers. (Table 1)

Clinical attachment loss: There was no statistically significant difference in the clinical attachment loss between smokers and non-smokers (Table 1)

Table 1: Intra group comparison of the various clinical parameters

Group	N	Mean	P
PI: Smokers	50	1.7112	0.079
Non smokers	50	1.9002	
GI: Smokers	50	1.1730	0.043
Non smokers	50	1.3202	
BOP: Smokers	50	1.9076	0.017
Non smokers	50	2.4658	
PPD: Smokers	50	2.9888	0.181
Non smokers	50	3.2786	
CAL: Smokers	50	2.8152	0.382
Non smokers	50	3.0156	

Table 2: Shows the bacterial load among the groups

BACTERIAL LOAD	SMOKERS	NON-SMOKERS
1	10	5
2	14	18
3	16	16
4	10	11
Total	**50**	**50**

There was no statistically significant difference in the P.gingivalis bacterial counts among the smokers and non smokers. P= 0.147 (Table 2)

Table 3: Co-relation between the various clinical parameters and bacterial load in both groups

		Porphyromonas Gingivalis
PI	Pearson Correlation	.630
	Sig. (2-tailed)	.000
	N	100
GI	Pearson Correlation	.
	Sig. (2-tailed)	.
	N	100
BOP	Pearson Correlation	.
	Sig. (2-tailed)	.
	N	100
PPD	Pearson Correlation	.830
	Sig. (2-tailed)	.000
	N	100
CAL	Pearson Correlation	.664
	Sig. (2-tailed)	.000
	N	100

A positive co relation was found between the increasing probing pocket depth and clinical attachment loss with the P.gingivalis counts. Also there was found a positive co relation between increasing plaque scores and increasing P.gingivalis counts.

DISCUSSION

The present study aimed at finding the bacterial load of *Porphyromonas gingivalis* and correlating with clinical status in chronic periodontitis patients. The study also evaluated the impact of smoking on *Porphyromonas gingivalis.*Conventional methods for identification of *Porphyromonas gingivalis* is difficult, laborious and time consuming. It needs anaerobic culture set up with the necessary identification tests.The newer molecular methods has made the identification of organisms easier in a shorter span of time.

The only clinical difference between smokers and non-smokers was in the gingival index and bleeding on probing. No difference in the mean probing pocket depth, clinical attachment loss and *P.gingivalis* counts between smoker and non-smoker patients was seen.

The present study found no difference in the plaque scores between smokers and non smokers. This finding is in accordance with (Palmer *et al.*, 2005 and Bergstrom, 1981) who also have found that, when controlling for other factors, smoking did not appear to increase the amount of plaque formation in smokers. However, earlier studies demonstrated that smokers showed a higher prevalence of dental plaque than non-smokers (Novak).

Smokers had statistically significant lesser gingivitis scores and reduced bleeding on probing compared to non smokers. This is in accordance with the past literature (Tonetti, 1998) where evidences have been given by using both the gingival index and a dichotomous evaluation of bleeding on probing. Furthermore, development of gingival inflammation in response to experimental plaque accumulation (experimental gingivitis) was less pronounced in smokers than in non-smokers (Bergstrom, 1981). These cross sectional and longitudinal data are generally interpreted as an indication that cigarette smoking is an environmental exposure which is able to modulate gingivitis expression in response to dental plaque.

No statistically significant difference was detected in the Probing pocket depth and clinical attachment level among smokers and non-smokers. A positive correlation was found between the increasing probing pocket depth and clinical attachment loss with the *P.gingivalis* counts. A positive correlation was found between increasing plaque scores and increasing *P.gingivalis* counts. Limited influence of smoking on periodontal pathogens has been reported earlier. Among them, Bostrom *et al.*, (2001) found that the detection rate of periodontopathic bacteria was higher in smokers, although not significantly. Haffajee and Socransky (2001) reported that species such as *T. forsythia, P. gingivalis, T. denticola, P. intermedia, P. micra* and *P. nigrescens* were significantly higher in the current smokers at sites with pocket depth < 4 mm compared with the non-smokers

The present cross-sectional investigation assessed the clinical status and the subgingival *P.gingivalis* counts in smokers compared to non-smokers. The results showed that smokers have similar periodontal health compared to non-smokers; specifically, in terms of CAL and PPD. Instead the PPD, CAL and subgingival *P.gingivalis* count was positively co related to the amount of plaque present.

In most labs, identification of *P.gingivalis* is done by biochemical tests, which are laborious, time consuming, cumber some and also not reliable. In contrast to this, we here present a method which includes both bacterial culture and PCR. Thus, it is more accurate, rapid, sensitive and specific as it targets the specific genetic probes/primers in the identification of micro organisms.

Conclusion

P. gingivalis is a well-recognized potential periodontopathogen. The PCR technique is sensitive and bacterial culture being the gold standard for the growth and identification of P.gingivalis. The validity and accuracy of results of microbiological analysis are also influenced by the ability of the method to reliably measure the presence and frequency of bacteria in collected samples. Thus, the present study combined both the microbiological and molecular technique to assure a more accurate and rapid method of detection and found that there is no statistically significant difference in the *Porphyromonas gingivalis* load in the subgingival plaque among smokers and non smokers.

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