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Original Article

Dentistry Section

Quantification of *Selenomonas sputigena* in Chronic Periodontitis in Smokers Using 16S rDNA Based PCR Analysis

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ABSTRACT

Background and Aim: *Selenomonas* species have been associated with chronic periodontitis and have been implicated in converting periodontal health to disease. Scanty literature is available in Indian population. Hence, the objective of the study was to detect the prevalence of *Selenomonas sputigena* in healthy and chronic periodontitis by polymerase chain reaction (PCR) in Indian population and to check whether smoking affects the subgingival microflora of this organism in chronic periodontitis.

Materials and Methods: A total of 60 subjects with severe chronic periodontitis with or without smoking and periodontal healthy subjects underwent clinical and microbiological assessment. A deep subgingival plaque sample was collected and genomic DNA was extracted from each sample and analysed for detection of *Selnomonas sputigena* using PCR. The frequency and quantification of bacteria were also estimated.

Results: All groups differed statistically significant in the frequency of detection of *Selenomonas sputigena*. On comparison of patients with chronic periodontitis in smokers and non-smokers, there was no statistically significant difference. When the results were quantified, statistically non-significant results were seen among all groups. Plaque index, gingival index, probing pocket depth and clinical attachment level were statistically non-significant in chronic periodontitis with smokers and non-smokers.

Conclusion: Prevalence of *Selenomonas sputigena* showed significant differences with respect to the frequency of detection when comparing the disease group to the healthy population. But no significant difference was seen when the results were quantified. Smoking has no influence on number of *Selenomonas sputigena*. This study highlights presence as well as quantity of the organism is very important in elucidating its role in causation and progression of the disease.

Keywords: Molecular biology/polymerase chain reaction, Microbiology/*selenomonas sputigena*, Smoking

INTRODUCTION

Selenomonas sputigena (S. sputigena) is one of the frequently detected obligatory motile, gram negative, anaerobic, non-spore forming bacillus in dental plaque. It has been implicated as an etiological agent in periodontal disease [1,2]. *S. sputigena* can be isolated and correlated with other subgingival microflora [3,4]. The subgingival microflora of generalized chronic and aggressive periodontitis exhibited higher proportions *Selenomonas* species [5,6]. The most numerous species in established periodontitis belonged to genera *Selenomonas*, using 16s clonal analysis [7].

The isolation and detection of *Selenomonas* species from dental plaque by conventional methods is difficult due to their fastidious obligatory anaerobic characteristics. However, it is still necessary to identify its etiopathological role in periodontal diseases /health and no information is available about its absolute number and proportions in distinguishing healthy and diseases sites and its response to therapy. Molecular techniques have overcome many limitations of microbial culture methods [8]. The quantitative polymerase chain reaction (PCR) technique is a simple with high specificity and sensitivity, can detect fewer than 10 *S. sputigena* cells [9]. The quantitative PCR using species-specific oligonucleotide primers can enhance the rapid detection and enumeration of *Selenomonas* species. The identification of *S. sputigena* is now made easy by designing species-specific sequences of their16S rDNA sequences [2]. The subgingival microflora in smokers is different from healthy or non-smokers. The subgingival flora is characterized by highly diverse and unstable colonization of plaque biofilm. Further evidence of an altered host-bacterium interaction in smokers was obtained by robust correlation between the genus *Selenomonas* and the proinflammatory response [10,11].

The role of *S. sputigena* is well established in aggressive periodontitis, its role in chronic periodontitis is still not clear. Scanty literature is available on its prevalence in chronic periodontitis. Thus, the present study is to find the prevalence and quantification of *S. sputigena* levels in chronic periodontitis patients by using 16s rDNA PCR in Indian population. This study also aimed at comparing whether smoking affects the subgingival microflora in smokers with regards to the *S. sputigena*.

MATERIALS AND METHODS

This cross-sectional prevalence study carried out within a span of one year comprised of 60 subjects divided into 3 groups: 30 healthy and 30 chronic periodontitis (15 smokers and 15 nonsmokers). All subjects were recruited consequently from Department of Periodontology, Sinhgad Dental College and Hospital, Pune, India, after considering inclusion and exclusion criteria's. Healthy patients were nonsmokers and had sites with probing depth and clinical attachment loss not more than 3mm and less than 10% sites exhibiting bleeding. Nonsmoker chronic periodontitis patients (NCP) had probing pocket depth (PPD) and clinical attachment loss (CAL) ≥ 5mm, visible plaque, presence of gingival bleeding. Further, individual who smoked more than 20 cigarettes at the time of study [11] were considered as current smokers were included in smoking chronic periodontitis (SCP) group. Any systemic conditions that could affect the progression of periodontal disease, pregnancy & lactation, periodontal therapy in the last 12 months, administration

of any anti-inflammatory and antibiotic therapy in the previous 3 months were excluded from the study. All patients were informed of the purpose and protocol of the study and all the concerns raised were answered to their satisfaction. An informed consent was taken prior to the procedure.

Clinical Parameters: Plaque index (PI) by Turesky, Gilmore, Glickman [12], Gingival index (GI) by Loe and Silness [13], were evaluated clinically. Measurements of PPD were made at four sites per tooth (buccal/facial, mesial, distal, and lingual/ palatal surfaces) with the help of a Williams graduated periodontal probe. The CAL is measured from the base of the pocket to cementoenamel junction.

Collection of plaque sample for DNA extraction: Individual subgingival plaque sample was collected by sterile Gracey curette [14] from three deepest subgingival sites of CAL of ≥ 5mm for chronic periodontitis patients and from three sites of CAL ≤ 3mm in healthy patients. Each of the target teeth was isolated with cotton rolls and air dried after removal of supragingival plaque and calculus before collecting subgingival plaque sample. Individual subgingival plaque sample were immediately placed in eppendorf tube containing TE buffer. The tube was sealed and transported to laboratory within 24 hours. Within 48 hours DNA was extracted.

DNA Extraction - (Modified Proteinase-K method) [2]

The tube was centrifuged at 5,000 rpm for 5 min. The supernatant that was formed was discarded. 500 µl fresh T.E. buffer was added and centrifuged for 3-4 min. The above procedure was repeated for 3-4 times with fresh T.E. buffer. Supernatant was discarded and 50 µl lysis buffer I was added, vortexed and kept for 5 mins, 50 µl lysis buffer II and 10 µl proteinase – K (100 µg/ml) was added and vortexed vigorously. It was kept in water bath for 2 hours and then in boiling water bath for 10 min. The resultant sample that was obtained was DNA which was stored at -20^{0}C. The purity of DNA was checked by electrophoresis.

PCR Amplification: PCR Master Mix (Chromous Biotech Pvt. Ltd, Bangalore, India) was used containing 10X Taq polymerase Buffer containing 1.5mM $MgCl_2$ and 10mM dNTP mix, and 1.5U/reaction Taq polymerase enzyme. Twenty five µl of above master mix was taken to thermostable PCR tubes, 1.2 µl each of Forward and Reverse primers (Bioserve Biotech India Pvt. Ltd, Hyderabad, India.) specific for *S. sputigena* was added (30pmole). 5 µl Template DNA (<100 ng) was added and finally the total volume was made to 50 µl by adding water. Primer sets R8F: 5' -AGAGTTTGATCCTGGCTCAG -3' and SS-R: 5' -CTCAATATTCTCAA GCTCGGTT -3'were used for specifically amplifying *S. sputigena*. The tubes were kept in a thermal cycler (Applied biosytem, California, USA) for carrying out initial denaturation (95^{0}C, 5min) followed by denaturation (95^{0}C, 1min), annealing (54^{0}C, 30 sec) and extension (72^{0}C, 1min) repeated for 35 cycles before final extension (72^{0}C for 5 min) to allow all extensions to be completed for amplification. Samples were kept at 4^{0}C following PCR method.

Detection of Amplified Products: Amplified products were subjected to electrophoresis for size separation of the PCR products. 1.5% agarose gel (Qiagen, Bangalore, India) containing 1x TAE, 20 µl of each amplified product was mixed with 3 µl of bromophenol blue loading dye. Electrophoresis was performed at 25V for 2 hours. The gel was visualized under UV light illuminator after staining with ethidium bromide (0.5µg/ml). The quantification was done based on the band intensity of the test samples compared with the 100 bp DNA ladder which was run simultaneously with the samples. The markers were seen on monitor screen. Total Lab Quant software (Total Lab, UK) was used to quantify the data.

STATISTICAL ANALYSIS

Data was compiled on excel sheet and SPSS software 17 (SPSS Inc. Chicago, USA) was used for statistical analysis. T-test was used for comparison of clinical parameters with gender. Statistical analysis for comparison between all the groups was done with ANOVA followed by post hoc test for intergroup comparison. Chi-square test was used to compare the detection frequencies of bacteria between all the groups and intergroup comparison. Mann Whitney U-test was used for quantitative assessment of bacteria for intergroup comparison. p-value < 0.05 was considered to be statistically significant.

RESULTS

Out of 60 patients, 15 were non-smokers with chronic periodontitis, 15 were smokers with chronic periodontitis and 30 were healthy controls. The mean age of the patient was 42 ± 8.543 y (range 34-68 y). Out of them 40 were males and 20 were females.

The pre-procedural clinical assessment was done using PI and GI. On comparison of PI, and GI with gender there was non-statistically significant difference [Table/Fig-1]. Comparison of PI and GI in all groups (NCP, SCP, Healthy) by ANOVA [Table/Fig-2] resulted in a statistically significant difference (p<0.001).

Intergroup comparison of PI in NCP and SCP groups were statistically significant compared to healthy, but statistically non-significant between NCP and SCP group. GI was statistically significant in all groups. GI, PD and CAL in subjects with chronic periodontitis in smokers and non-smokers had a statistically significant difference. [Table/Fig-3,4].

All three groups differed statistically significant in the frequency of detection of *S. sputigena* (p<0.001) showing highest prevalence in chronic periodontitis group [Table/Fig-5]. Similarly, prevalence of occurrence of *S. sputigena* in NCP group was highest (66%; 10/15) compared to SCP group 33% (5/15) and least in healthy group 13% (4/30).

When patients with chronic periodontitis without smoking habit (NCP) were compared with smoking habit (SCP), there was no statistically

Clinical parameter	Sex	Mean	Std. Deviation	p - value
PI	Male	2.34	0.803	0.161
	Female	2.01	0.906	
GI	Male	0.94	0.773	0.186
	Female	0.65	0.783	
PPD	Male	3.77	3.409	0.102
	Female	2.25	3.188	
CAL	Male	3.84	3.474	0.090
	Female	2.25	3.188	

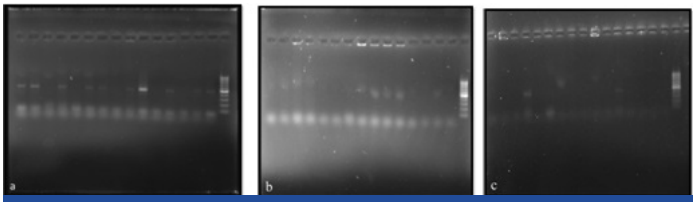
[Table/Fig-1]: Comparison of plaque index, gingival index, probing pocket depth and clinical attachment level with gender
PI: plaque index, GI: gingival index, PPD: probing pocket depth and CAL: clinical attachment level

Clinical parameter	Group	Mean	Std. Deviation	Std. Error	p- value of ANOVA
PI	NCP	2.61	0.549	0.142	< 0.001*
	SCP	2.91	0.594	0.153	
	Healthy	1.69	0.725	0.132	
GI	NCP	1.75	0.363	0.094	< 0.001*
	SCP	1.34	0.454	0.117	
	Healthy	0.14	0.094	0.017	
PD	NCP	6.90	1.156	0.299	< 0.001*
	SCP	6.15	1.056	0.273	
	Healthy	0.00	0.000	0.000	
CAL	NCP	7.01	1.177	0.304	< 0.001*
	SCP	6.23	1.051	0.271	
	Healthy	0.00	0.000	0.000	

[Table/Fig-2]: Comparison of plaque index, gingival index, probing pocket depth and clinical attachment level with all groups
PI: plaque index, GI: gingival index, PPD: probing pocket depth and CAL: clinical attachment level, NCP: non-smokers chronic periodontitis, SCP: smokers chronic periodontitis. *p< 0.05

Dependent Variable	Groups (I)	Groups (J)	Mean Difference (I-J)	Std. Error	p-value of Post Hoc test
PI	NCP	SCP	-0.293	0.239	0.442
	SCP	Healthy	1.214	0.207	< 0.001*
	Healthy	NCP	-0.921	0.207	< 0.001*
GI	NCP	SCP	0.412	0.108	0.001*
	SCP	Healthy	1.202	0.094	0.001*
	Healthy	NCP	-1.615	0.094	< 0.001*
PPD	NCP	SCP	0.757	0.283	0.026*
	SCP	Healthy	6.146	0.245	< 0.001*
	Healthy	NCP	-6.903	0.245	< 0.001*
CAL	NCP	SCP	0.782	0.286	0.022*
	SCP	Healthy	6.233	0.247	< 0.001*
	Healthy	NCP	-7.015	0.247	< 0.001*

[Table/Fig-3]: Intergroup comparison of plaque index, gingival index, probing pocket depth and clinical attachment level
PI: plaque index, GI: gingival index, PPD: probing pocket depth and CAL: clinical attachment level
NCP – non-smokers chronic periodontitis; SCP-smokers chronic periodontitis. *p< 0.05



[Table/Fig-4]: Identification of *Selenomonas sputigena* by gel electrophoresis
a - Nonsmoking chronic periodontitis subjects (10/15).
b - Smoking chronic periodontitis subjects (5/15).
c - Healthy subjects (4/30).

		NCP	SCP	Healthy	p-value of Chi Square test
S.S	Presence	10	5	4	0.001*
	Absence	5	10	26	
	Total	15	15	30	

[Table/Fig-5]: Frequency and prevalence of occurrence of *Selenomonas sputigena* in all the groups
NCP – non-smokers chronic periodontitis; SCP-smokers chronic periodontitis. *p< 0.05

		NCP	SCP	p-value of Chi Square test
S.S	Presence	10	5	0.068
	Absence	5	10	

[Table/Fig-6]: Frequency of occurrence of *Selenomonas sputigena* in smokers and nonsmokers chronic periodontitis groups
NCP – non-smokers chronic periodontitis; SCP-smokers chronic periodontitis

		NCP	Healthy	p-value of Chi Square test
S.S	Presence	10	4	< 0.001*
	Absence	5	26	

[Table/Fig-7]: Frequency of distribution of *Selenomonas sputigena* in nonsmokers chronic periodontitis and healthy groups
NCP – non-smokers chronic periodontitis, *p < 0.05

	N	Mean	Std. Deviation	p-value of ANOVA
NCP	10	58810.00	128352.609	0.078
SCP	5	39200.00	29175.332	
Healthy	4	19575.00	28475.530	

[Table/Fig-8]: Quantification of *Selenomonas sputigena* in all the groups
NCP – non-smokers chronic periodontitis; SCP-smokers chronic periodontitis

significant difference in prevalence of subgingival sample of *S. sputigena* (p= 0.068) [Table/Fig-6]. However, statistically significant difference was observed when NCP subjects were compared with healthy subjects (p < 0.001) [Table/Fig-7].

Quantification of *S. sputigena* in all the groups showed statistically non-significant difference (p=0.078) [Table/Fig-8]. Comparison

	Groups	N	Mean	Std deviation	p-value of Mann Whitney u-test
NCP v/s SCP	NCP	10	58810.00	128352.609	0.270
	SCP	5	39200.00	29175.332	
NCP v/s Healthy	NCP	10	58810.00	128352.609	0.572
	Healthy	4	19575.00	28475.530	

[Table/Fig-9]: Quantification of *Selenomonas sputigena* in between nonsmokers chronic periodontitis and smokers chronic periodontitis groups and nonsmokers chronic periodontitis and healthy groups
NCP – non-smokers chronic periodontitis; SCP-smokers chronic periodontitis

of NCP with SCP group and NCP with healthy group resulted statistically non-significant difference (p=0.270 and p=0.572 respectively) [Table/Fig-9].

DISCUSSION

The present study was conducted to find out prevalence and quantification of *S. sputigena* in chronic periodontitis in smokers and non-smokers in Indian population using 16s rDNA analysis by quantitative PCR. The 16S rRNA gene represents a highly conserved, accurate and versatile method for bacterial identification. Several novel oral bacterial species are detected by this technique [15-17]. Oral members of the *Selenomonas* species have repeatedly been associated with periodontal disease [18]. *S. sputigena* have been detected in periodontal pockets, but not in healthy gingival sulci using PCR suggesting that it may have the potential to be involved in pathogenesis of perioodontitis [6,11,19-26].

The prevalence levels that have been determined in different studies vary considerably. While some authors concluded high prevalence rates between 70% and 100% for different *Selenomonas* isolates and clones [23,24]. Other groups find the prevalence to be below 30% in both chronic periodontitis and generalized aggressive perioodontitis [21]. In several studies reporting high prevalence for diseased groups, prevalence of up to 70% are also determined for the respective healthy control groups [24,27]. Other authors find highly significant differences between healthy and affected sites and suggest *Selenomonas* species to be appropriate diagnostic markers for active periodontal disease [28]. Thus the prevalence of *S. sputigena* in chronic periodontitis and healthy subjects is still confusing and need a better understanding to elucidate its role in pathogenesis of the disease.

The prevalence of *S. sputigena* in the present study was 66% and 33% in chronic periodontitis without smoking and with smoking respectively and least 13% in healthy group. These values were statistically significant showing higher prevalence in chronic periodontitis group. These results were similar to study done by Habashneh in Jordanian population [19] and Kumar et al., [24]. But Mayanagi et al., [25] reported that *S. sputigena* seemed to be higher in subgingival plaque samples from periodontitis subjects than in healthy subjects, although these differences were not statistically significant by multiple comparisons. Similarly Charalampakis et al., [26] using DNA–DNA hybridization method observed that *S. sputigena* proved to be an irrelevant target group because they were undetectable in most investigated sites in periodontitis patients at the different study time points. This suggests that it is highly unlikely that *S. sputigena* contribute to the disease process and may have a potential role in chronic periodontitis.

It has been suggested that modification of the periodontal microflora by smoking is involved in the development of periodontitis. No study is yet done to detect the effect of smoking on *S. sputigena* in chronic periodontitis. Hence, the present study also aimed to compare the prevalence of *S. sputigena* in chronic periodontitis with smokers and non-smokers. Though significant difference was noted in clinical parameters, microbiological reports were non-significant in either group. In early onset periodontitis, Kamma et al., [11] reported that smokers harboured higher level of *S. sputigena* compared to nonsmoker. Other study found that both smokers and

non-smokers exhibited similar subgingival microflora, suggesting that smoking has limited influence on the microflora involved in periodontal disease [29]. Our study indicated that smoking does not affect its presence in subgingival area. Considering these variations, more information is needed to delineate the relationship between smoking and periodontal microflora.

In the present study, the quantification of the presence of Ss was done with Total Lab software, UK. It was observed in this study that though presence of microorganism was significant in chronic periodontitis compared to healthy, quantification value was not significant. This observation is similar to study done by Kumar et al., [24]. This signifies that the presence of a particular microorganism though important, the quantity in which it is present is also necessary for causation and progression of disease. It was also observed in this study that Ss were present in healthy subjects, suggesting that they may also be regarded as normal plaque commensal. The sampling technique might also have influenced the bacterial quantification.

Despite its possible association with disease like necrotizing ulcerative perioodontitis [30], aggressive [11] and chronic perioodontitis [19,24] the components and the products of *Selenomonas sputigena* involved in the colonization of the oral cavity and the mechanisms of inducing tissue destruction are unknown. In a study done by Drescher et al., [1] on molecular epidemiology and spatial distribution of *Selenomonas* species it was observed that *Selenomonas* spp. appeared in large numbers in all parts of the collected biofilms and seemed to make a relevant contribution to their structural and architectural organization and plays a very important role in co-aggregation and maturation of plaque. It induces bone resorption in in-vivo experimental animal [31]. Composition of lipid A appears to be an important virulence factor and is able to induce interleukin-1α and interleukin-6 in murine macrophages thus suggesting its role in inflammation [30,32].

However, some limitations of this study should be considered. These include its cross-sectional design, and small sample size. Despite these limitations, this study provides valuable information on *Selenomonas sputigena* in an Indian population, which may be useful in the establishment of preventive and therapeutic strategies suitable for people in India. Further investigations can be carried out on large sample size and with different periodontal disease groups as well as the viable count of the microbe can be assessed at disease site by cultural test. The correlation of microbiological and clinical parameters with periodontal disease activity is difficult to ascertain. Any identification and analysis of the microorganisms associated with different forms of periodontitis must take into account the stage of disease activity in the site, at that point of time. In addition, a variety of factors like age of the patient, age and maturation of plaque, diet, host response, adverse habits, hormonal activity, antibiotics may alter the environment and the outcome of any study.

CONCLUSION

Prevalence of *S. sputigena* showed significant differences with respect to the frequency of detection when comparing the disease group to the healthy population. But no significant difference was seen when the results were quantified. This shows that presence as well as quantity of the organism is very important in elucidating its role in causation and progression of the disease. Though smoking affects the subgingival microbial profile of many periodonto pathogens, no significant difference was observed between smokers and non-smokers in chronic periodontitis. *S. sputigena* may be associated with the pathogenesis of chronic periodontitis and therefore their role in the onset and progression of this infection merits further investigation.

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