Detection of *Filifactor alocis* and *Dialister pneumosintes* in Biofilms of Smokers and Nonsmokers with Chronic Periodontitis: A PCR Study

Shwetha Eshwarappa¹, Chethana K Chidambar², Shrinidhi M Shankar³

ABSTRACT

Aim and objective: Detection of *Filifactor alocis* and *Dialister pneumosintes* in smokers and nonsmokers with chronic generalized periodontitis.

Materials and methods: Out of 60 patients diagnosed with chronic generalized periodontitis, 30 current smokers and 30 nonsmokers were recruited for the study. Clinical parameters such as gingival bleeding index, plaque index, and probing pocket depth were recorded. Subgingival plaque samples were collected and subjected to a multiplex polymerase chain reaction (PCR) assay.

Results: Collected plaque samples of nonsmokers with chronic periodontitis patients showed a slight increase in numbers of *F. alocis* and *D. pneumosintes* species when compared with smokers, although statistically not significant.

Conclusion: The distribution of *F. alocis* and *D. pneumosintes* which are considered as the masked pathogens of the periodontal disease was not significantly different in nonsmokers and smokers with chronic periodontitis.

Clinical significance: These species which were rarely studied can be considered as markers for periodontal disease, as they denote increased periodontal destruction.

Keywords: Biofilm, *Dialister pneumosintes*, *Filifactor alocis*, Polymerase chain reaction, Smokers.

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INTRODUCTION

Periodontal diseases are a result of specific microbial complexes in subgingival biofilms.¹ Polymicrobial associations which exhibit structural and functional heterogeneity are termed biofilms. Periodontitis is a dysbiotic disease wherein there is a shift in the microbial profile from a gram-positive toward gram-negative² or more from orange to red complex, thus gradually changing the symbiotic host-microbe relationship to a more pathogenic one. Contrary to this microbial association with the disease, recent studies have proven the association of other organisms like *Veillonella* species. However, these pathogens have not been isolated or proven to have a direct role in the etiology of periodontitis.³ Some of the new organisms associated with periodontal disease are *Filifactor alocis* and *Dialister pneumosintes*.

Filifactor alocis is a fastidious, gram-positive, obligatory anaerobic rod that possesses trypsin-like enzymatic activity similar to *Porphyromonas gingivalis* and *Treponema denticola*. In comparison to the other traditional periodontal pathogens, the high incidence of *F. alocis* in the periodontal pocket compared with its absence in healthy or periodontitis-resistant patients has highlighted its importance in the infectious disease process. It has also been identified as one of the prevalent phylotypes in cases of failed endodontic treatment and associated with peri-implantitis. *Filifactor alocis* has a unique property such as resistance to oxidative stress and genes coding for a well-developed amino acid metabolic pathway, which can allow it to colonize and polymicrobial synergistic relationship which can enhance its invasive capacity and survive with other traditional periodontal pathogens in the stress environment of the periodontal pocket. *Filifactor alocis* was detected in elevated numbers in chronic periodontitis patients and was therefore proposed as a potential marker for active disease.⁴

¹⁻³Department of Periodontics, Sharavathi Dental College and Hospital, Shivamogga, Karnataka, India

Corresponding Author: Chethana K Chidambar, Department of Periodontics, Sharavathi Dental College and Hospital, Shivamogga, Karnataka, India, Phone: +91 9448168733, e-mail: anuhgd@gmail.com

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Dialister pneumosintes, a resident of the upper respiratory tract and oral cavity, is a small, non-fermentative, gram-negative anaerobic rod, which has been isolated from periodontal lesions and endodontic infections. It is often associated with other bacterial pathogens and viruses in mixed periodontal and root canal infections.¹

Chronic periodontitis results from the presence of complex microbial communities in the subgingival sulcus influenced by smoking, a major environmental factor associated with the development of extensive and severe periodontal disease. One of the first ecosystems to come into contact with tobacco smoke, and to be affected by it, is the oral microbiome. Smokers present a high-at-risk cohort for periodontitis, a microbially driven oral disease that affects two-thirds of adult humans. Smokers acquire these pathogens within 24 hours of biofilm formation and are associated with pathogen enrichment.⁵

The present study aimed to detect the presence of *F. alocis* and *D. pneumosintes* in the subgingival plaque of smokers and

nonsmokers diagnosed with chronic periodontitis by using the polymerase chain reaction (PCR) technique.

MATERIALS AND METHODS

A total of 60 patients who required periodontal treatment were recruited from the Outpatient Department of Periodontology, Sharavathi Dental College and Hospital, Shivamogga. Patients diagnosed with chronic generalized periodontitis as per the AAP 1999 criteria were grouped into 30 current smokers⁶ and 30 nonsmokers. The study was conducted in full accordance with ethical principles and was approved by the institutional ethics committee (Ref no:-SDC/SMG/2017/649) and also written informed consent was obtained from each patient. Patients in the age group of 35–55 years with at least 20 natural teeth and a minimum of 6 teeth with periodontal pockets with a depth of ≥ 4 mm were included.⁷ Exclusion criteria included patients who had a history of antibiotic use or periodontal treatment 6 months before the study or with systemic conditions known to affect the periodontal status, immuno-compromised individuals, pregnant, and lactating women. Clinical examination was done by a single examiner using William's periodontal probe. Plaque index,⁸ gingival bleeding index,⁹ and probing pocket depth (PPD) were recorded. The patients were divided into two groups; Group I comprised 30 nonsmokers while group II comprised 30 current smokers. This study was cross-sectional and observational in nature, hence no controls were included.

Method of Collection of Sample and Microbial Analysis

The subgingival plaque samples were collected before the initial phase of periodontal therapy. The samples were collected by inserting a sterile curette into the periodontal pockets. Later, it was pooled in 50 μ L TE transport medium (Tris-HCl, Tris-EDTA, and distilled water) and the samples were sent to the laboratory for PCR analysis. Multiplex conventional PCR was carried out for the detection of *D. pneumosintes* and *F. alocis* by using DNA extracts (Template) PCR master mix (Ampliqon red) and species-specific PCR primers such as forward primer: CAG GTG GTT TAA CAA GTT AGT GG [Bioserve Biotechnologies (India) Pvt Ltd, Hyderabad] and reverse primer: CTA AGT TGT CCT TAG CTG TCT CG with base pair of 594 for *F. alocis* and forward primer: TTC TAA GCA TCG CAT GGT GC [Bioserve Biotechnologies (India) Pvt Ltd, Hyderabad] and reverse primer: GAT TTC GCT TCT CTT TGT TG with base pair of 1,105 for *D. pneumosintes*. Initial denaturation was done at 95°C for 5 minutes followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. The final extension was carried out at 72°C for 5 minutes. Post-PCR analysis was done by running the amplified products on 2% agarose gel electrophoresis. The PCR products were loaded into 2% agarose gel matrix and electrophoresis was run at 75 V for 2 hours. The gel was stained with ethidium bromide and observed under a UV transilluminator. Photos were captured using the gel doc system. The bands obtained for each sample were compared with the DNA ladder and a specific band of organism was identified (Fig. 1).^{10,11}

STATISTICAL ANALYSIS

The results obtained from various parameters were subjected to statistical analysis. Results were expressed as mean with standard deviation. Intergroup comparisons were done by using the

Fig. 1: Representative PCR amplicons. Clinical samples are positive for the presence of bacteria after amplification by multiplex PCR for *Filifactor alocis* and *Dialister pneumosintes*. Clinical samples are positive for *D. pneumosintes*. (Lane 1, 7) Clinical samples are positive for *F. alocis*. (Lane 7) Clinical samples are positive for both species. (Lane 7)

Chi-square test and independent *t*-test. For all the tests, a *p* value of 0.05 or less was considered for statistical significance.

RESULTS

On gender distribution, 24 were males and 6 were females in group I whereas 27 were males and 3 were females in group II. No difference was seen with the Chi-square with Yates's correction = 0.5231, *p* = 0.4701 (Table 1).

The mean age in subjects with smokers was 47.43 with a standard deviation of 4.68 and in subjects with nonsmokers were 45.93 with a standard deviation of 6.00. No significant difference was observed between group I and group II with mean age (*t* = 1.0797, *p* = 0.2848; Table 1).

The mean plaque index score in subjects with smokers was 1.60 with a standard deviation of 0.22 and in subjects with nonsmokers was 1.51 with a standard deviation of 0.21. No significant difference was observed between group I and group II with mean plaque index scores (*t* = 1.6798, *p* = 0.0984; Table 2).

The mean gingival bleeding index score in subjects with smokers was 0.65 with a standard deviation of 0.14 and in subjects with nonsmokers was 0.35 with a standard deviation of 0.11 significant difference was observed between group I and group II with mean gingival bleeding scores (*t* = 9.4126, *p* = 0.0001). The mean gingival bleeding index was significantly higher in group I as compared to group II (*p* = 0.0001 and Table 3).

The mean probing depth score in subjects with smokers was 6.11 with a standard deviation of 0.60 and in subjects with nonsmokers were 6.07 with a standard deviation of 0.68. No significant difference was observed between group I and group II with mean probing depth scores (*t* = 0.2671, *p* = 0.7904). The mean probing depth scores were similar in group I and group II (Table 4).

In the present study, the presence of *D. pneumosintes* and *F. alocis* was determined by multiplex PCR amplification of reference genomic DNA. Primer pairs were used for the identification of two pathogens. Clinical samples that were positive for *F. alocis* and/or *D. pneumosintes* also showed one band of the predicted size per species and the absence of non-specific amplification products.

Table 1: Comparison of nonsmoker and smoker with mean age by independent *t*-test and distribution of male and females in nonsmoker and smoker

Groups	Age				Sex			
	Mean	SD	SE	<i>p</i> value*	Male	Female	Total	<i>p</i> value*
Nonsmoker	47.43	4.68	0.85	0.2848	24	6	30	0.4701
Smoker	45.93	6.00	1.10		27	3	30	

SD, standard deviation; SE, standard error

*The mean difference is significant at the 0.05 level; NS, not significant

Table 2: Comparison of nonsmoker and smoker with plaque index scores by independent *t*-test

Groups	Mean	SD	SE	<i>t</i> value	<i>p</i> value
Nonsmoker	1.60	0.22	0.04	1.6798	0.0984
Smoker	1.51	0.21	0.04		

SD, standard deviation; SE, standard error

The mean difference is significant at the 0.05 level

Table 3: Comparison of nonsmoker and smoker with gingival bleeding index scores by independent *t*-test

Groups	Mean	SD	SE	<i>t</i> value	<i>p</i> value
Nonsmoker	0.65	0.14	0.03	9.4126	0.0001*
Smoker	0.35	0.11	0.02		

SD, standard deviation; SE standard error

**p* < 0.05 indicates significant between them

Table 4: Comparison of nonsmoker and smoker with probing depth scores by independent *t*-test

Groups	Mean	SD	SE	<i>t</i> value	<i>p</i> value
Nonsmoker	6.11	0.60	0.11	0.2671	0.7904
Smoker	6.07	0.68	0.12		

SD, standard deviation; SE, standard error

The mean difference is significant at the 0.05 level

Table 5: Comparison of nonsmoker and smoker with presence status of *Dialister pneumosintes*

<i>Dialister pneumosintes</i>	Nonsmoker	%	Smoker	%	Total
Absent	12	40.00	19	63.33	31
Present	18	60.00	11	36.67	29
Total	30	100.00	30	100.00	60

Chi-square = 3.2701 *p* = 0.0711

The mean difference is significant at the 0.05 level

Table 6: Comparison of nonsmoker and smoker with presence status of *Filifactor alocis*

<i>Filifactor alocis</i>	Nonsmoker	%	Smoker	%	Total
Absent	16	53.33	21	70.00	37
Present	14	46.67	9	30.00	23
Total	30	100.00	30	100.00	60

Chi-square = 1.7636 *p* = 0.1846

The mean difference is significant at the 0.05 level

The DNA mixture of nonsmokers (8 samples) and smokers (15 samples) lacking DNA of the two target species yielded negative results in the multiplex PCR.

Dialister pneumosintes was identified in 60% (18 samples were positive) from nonsmokers subjects and 36.6% (11 samples were positive) from smokers out of the 30 samples. The prevalence of the organism was highest in the nonsmoker group when compared to the smoker group by using the Chi-square test with the value of 1.7636 and *p* value of 0.1846, which is not significant statistically (Table 5).

Considering all the collected samples, *F. alocis* was identified in 46.67% (14 samples were positive) from nonsmokers subjects and 30% (9 samples were positive) from smokers out of the 30 samples. The prevalence of the organism was highest in group I when compared to group B by using the Chi-square test with the value of 1.7636 and *p* value of 0.1846. While detection frequencies did not differ significantly between nonsmokers and smokers (Table 6); however, *D. pneumosintes* was seen more in numbers on intra comparison between the organisms in both the groups (Fig. 2).

Fig. 2: Comparison of nonsmoker and smoker with the presence of *Filifactor alocis* and *Dialister pneumosintes* by independent t-test

Clinically, subgingival plaque of participants with periodontitis has shown a higher prevalence of *D. pneumosintes* and *F. alocis* in the nonsmoker group rather than the smokers.

DISCUSSION

Periodontal diseases are classified as complex disorders where environmental, lifestyle, and genetic factors contribute to their etiology. The most important environmental factor is the diverse microbiota arranged in a biofilm, which acts as an initiator of periodontal destruction. Also, lifestyle factors such as stress, smoking, and oral hygiene habits act as modifiers of disease progression. Smoking is responsible for accelerated periodontal destruction and increased risk of periodontitis in younger adults, as an anaerobic environment promotes the growth of gram-negative periodontal pathogens in the subgingival plaque. However, on the contrary, a study using Gram staining techniques failed to show a significant difference in the subgingival microflora between smokers and nonsmokers.¹² Hence, a study done by Qiu et al.¹³ has suggested that smoking may affect the host response.

The present study used a multiplex conventional PCR protocol for simultaneous detection of *D. pneumosintes* and *F. alocis*. Multiplex conventional PCR allows the simultaneous detection of different bacterial species. This PCR assay showed high specificity because only DNA mixtures containing DNA from both species generated the expected amplicons (one per species) after multiplex PCR amplification. This gave the clinical samples positive or negative results for either *D. pneumosintes* and *F. alocis* or both. Positive cases showed only the species-specific bands and the absence of non-specific bands.

In the present study, the presence of *F. alocis* and *D. pneumosintes* in smokers were 30 and 36.61%, whereas in nonsmokers were 46.67 and 60%, respectively. Chronic periodontitis is a source of oxidative stress, leading to elevated numbers of *F. alocis* in these environments. The virulence factors of *F. alocis* allow it to persist in extreme oxidative stress situations.¹⁴ Furthermore, smoking changes the subgingival bacterial profile by reducing beneficial species and increase of treponemes,¹² and also has a direct inhibitory effect on the host response.¹³ It increases the oxidative stress quotient within the subgingival environment making it conducive for the growth of *F. alocis*. However, in the present study,

on the contrary, there was an increase in the number of both the organisms in nonsmokers with chronic periodontitis rather than smokers with chronic periodontitis.

These organisms are also associated with compromised pulp status,¹⁰ patients were reassessed for the pulp status of periodontally involved teeth using an electric pulp test. Consequently, 30% of teeth showed altered pulpal response suggestive of a developing endo lesion. Hence, the above findings substantiate the presence of endodontic pathogens in a periodontal pocket, even if the lesion is not a part of perio-endo continuum.¹⁵

Filifactor alocis as well as *D. pneumosintes* have been known to be endodontic pathogens. A study done by Siqueira and Rocas¹⁰ revealed more number of *D. pneumosintes* (50%) than *F. alocis* (40%) in endodontic infections. Similar results were found in the present study. The number of *D. pneumosintes* was found more in subgingival samples irrespective of smokers and nonsmokers when compared to the number of *F. alocis*.

Research is limited about the pathogenicity of *F. alocis*. *Filifactor alocis* produces FACIN a complement inhibiting substance similar to a dysbiotic infection caused by a keystone pathogen that alters the subgingival environment. This change leads to a complement lead inflammation where *F. alocis* inhibits all pathways and causes periodontal disease. A study done by Jansson et al. in the mid-90s have explored the role of endodontic pathogens in the progression of marginal tissue breakdown.¹⁵

The prominent presence of *D. pneumosintes* in the subgingival microbiota of periodontal disease has come to light only in the recent past. This microbe-associated frequently with endodontic infections, is also found to be associated with subgingival microbiota of aggressive periodontitis (AgP) patients.¹⁰ Earlier study done by Gomes et al. have shown *D. pneumosintes* and *F. alocis* were detected in higher numbers in deep pockets of smokers.¹⁶ However, data are limited about the virulence action of *D. pneumosintes*. The subgingival occurrence of *D. pneumosintes* might have been underestimated because of its strict anaerobic requirement and its slow growth. The high prevalence of *D. pneumosintes* in advanced periodontitis lesions and the apparent pathogenicity of the organism places it in the group of suspected periodontal pathogens.

The higher numbers of *F. alocis* and *D. pneumosintes* in CP nonsmokers than in CP smokers are of concern as it could be a passive increase or could have an actual contribution to the pathogenesis of the periodontal disease. This clinical significance could be attributed to the presence of undiagnosed endodontic lesions or teeth which needed endodontic attention along with the periodontal disease. *Filifactor alocis* is known to colonize polymicrobial biofilm communities. An *in vitro* community model showed the synergistic association of *F. alocis* and *Fusobacterium nucleatum*, whereas *Streptococcus gordonii* was an antagonist to the accumulation of *F. alocis*.¹⁷

Collectively, these observations showed that the pattern of *F. alocis* colonization will depend on the spatial composition of the microbial microenvironment. *Filifactor alocis* preferentially accumulates at the site rich in *Fusobacterium nucleatum*. On the other hand, presence of *Streptococcus gordonii*, its aggregation, and multiplication could have contributed to a decrease in the number of *F. alocis* in the CP smokers group.¹⁸ This complex synergistic and antagonistic interaction which occurs within the oral microbial community result in the success or failure of microbial colonization. Hence, colonization of *F. alocis* heavily depends on the inhabitant

of the microbial community. A study of pathogenicity, virulence factor, and colonization pattern of *F. alocis* and *D. pneumosintes* with other microbial communities is further required. The limited sample size and quantitative analysis could have been attributed to statistical insignificance in this study.

The present study evaluated an association between the presence of these microbes and periodontitis in smokers and nonsmokers and assessed whether to consider them as markers of periodontal disease. However, within the limitations of sample size, culture methods, and interventional trials, it can only be concluded that higher proportions of these novel organisms are associated with the progression of periodontal destruction and smoking contributes only as a risk factor and no role as a causative agent.

CONCLUSION

In the present study, even though there was no statistically significant difference between the groups, considering the limited sample size *F. alocis* and *D. pneumosintes* were noted in higher numbers in nonsmokers compared with smokers with chronic periodontitis. Hence, the use of these organisms as markers of periodontal disease with smokers should be considered with discretion. However, a higher presence of these masked pathogens could be considered as one of the contributing factors in periodontal disease pathogenesis and progression.

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