ORIGINAL ARTICLE

Prevalence of *Methanobrevibacter oralis* in Chronic Periodontitis Patients: A Pilot Study

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Abstract

Background: Periodontitis is polymicrobial infection. Literature evidences supports the use of 16S ribosomal RNA ribotyping method to detect more than 100 noncultivable bacterial species present in subgingival microflora, concluding "plaque is composed of nonspecific opportunistic pathogens" that induce species like *methanogenic archaea.* Archaea are found in the oral cavity of human in the saliva, oral biofilms, endodontic lesions, and subgingival deep periodontal pockets of periodontitis. Chronic periodontitis has been linked to multiple members of the domain bacteria; however, not one member explains its role in the periodontitis. There are few studies that linked archaea to the subgingival biofilm of chronic periodontitis. The purpose of this study is to determine the prevalence of archaea in periodontally healthy and chronic periodontitis.

Materials and methods: A total of 30 subjects (age range: 25–60 years), 15 subjects each in periodontally healthy and chronic periodontitis, participated in this study to find the prevalence of *Methanobrevibacter (M.) oralis*. Clinical parameters including probing pocket depth (PPD) and clinical attachment level (CAL) were recorded. The *M. oralis* was detected using the real-time polymerase chain reaction.

Results: The prevalence of archaea in chronic periodontitis is 40% and in healthy subjects 6.7%.

Conclusion: Chronic periodontitis showed more prevalence of archaea in periodontal pockets, which may suggest association with periodontitis.

Keywords: Archaea, Chronic periodontitis, Periodontally healthy, Polymerase chain reaction.

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Introduction

Periodontal diseases are polymicrobial infection and multifactorial in origin.[1,2] In the field of periodontology, the microbial cause of periodontal disease is studied extensively. In recent reports, using the 16S ribosomal RNA ribotyping method, it was found that there are more than 100 noncultivable bacterial species present in the subgingival biofilm, which are nonspecific and opportunistic species causing the disease[3] such as *methanogenic archaea* bacteria.[4]

Archaea are single-celled microbes that were first classified in 1977 as a new group of prokaryotes based on the ribosomal RNA gene sequencing.[5] The archaea that are found in the human and animals belong to the methanogen family and have the ability to produces methane from substrates, such as hydrogen and carbon dioxide. Archaea are found in the oral cavity of human including subgingival samples from deep periodontal pockets of periodontitis.[2] Detection of anaerobic archaea in subgingival periodontal pocket shows how these microbes can colonize the human host, their ability to colonize for longer terms, and how they coexist with endogenous flora in the host.

Many studies have demonstrated archaea in deep periodontal pockets, in different forms of periodontitis, isolated from deep pocket areas. However, they have not been detected in healthy patients.[5] The presence of archaea at the sites of periodontal disease can suggest the progression of periodontitis which is explained by the host genetics susceptibility to be colonized by oral methanogens that have the potential to compete with oral pathogens indicating their coexistence within the same environment. Thus, archaea indirectly promote periodontal disease in patients by serving as a hydrogen sink.[6]

A study done by Brusa et al. in 1986 isolated methanogen bacteria from subgingival plaque detected in 10 samples.[5] A study by Lepp et al. in 2004 showed the presence of archaea in chronic periodontitis by using a pair of primers targeting the SSU rRNA of archaea.[6] A study by Matarazoo et al. detected bacteria from 96.4% of subjects and 68% of sites in the generalized aggressive periodontitis group.[4]

Chronic periodontitis has been linked to multiple members of the domain bacteria; however, not one member explains the majority of the role of microbes in the periodontal disease. The microbial group has the ability to use hydrogen at low proportion, which alters the microbial ecosystem and may contribute in the shift of periodontal microbiota toward disease activity. Such organisms are archaea.[2]

There are few studies related to archaea with chronic periodontitis. Hence, in the present study, the archaeal 16S rRNA gene was detected in subgingival plaque samples of chronic periodontitis patients.

Materials and Methods

Subject Population

Subjects with chronic periodontitis and periodontally healthy individuals in the age group of 25–60 years were selected from the

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Department of Periodontics, College of Dental Sciences, Davangere, Karnataka (India). The study was approved by the institutional ethical committee. All subjects were given oral instruction about the procedure and a written informed consent was obtained.

A total of 30 subjects were divided into two groups as follows: group I—periodontally healthy ($n = 15$) and group II—chronic periodontitis ($n = 15$) were included in the study. Subgingival sites were selected based on complete intraoral examination including full-mouth periodontal probing and panoramic radiograph (orthopantomograph—OPG). One site was selected from each subject. In group I, the sulcus depth of up to 4 mm was included whereas in group II the minimum pocket depth (>8 mm) and clinical attachment loss (>5 mm) with radiographic evidence of bone loss were selected for subgingival plaque samples collection (in accordance with the Helsinki Declaration of 1975, as revised in 2002).

Inclusion and Exclusion Criteria

The criteria of selection were based on the following findings for each group: (1) periodontally healthy subjects without any bleeding on probing, pocket depth <3 mm, no bone loss, and no systemic conditions; (2) Chronic periodontitis subjects were selected based on the criteria of American Academy of Periodontology classification of periodontal diseases 1999,[1] which includes the following: had >20 natural teeth and exhibited at least 30% sites with pocket depth >8 mm and attachment level measurements >5 mm with radiographic (OPG) evidence of bone loss.

Exclusion criteria were as follows: history of any previous periodontal therapy, medications like antibiotics in the previous 6 months, pregnancy, nursing, smoking, radiation therapy, and any systemic diseases that may affect periodontal health.

Clinical Examination

Clinical examination was done for all subjects; probing pocket depth (PPD) (mm) and clinical attachment level (CAL) (mm) were recorded at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual) in all teeth. Pocket depth and CAL measurements were recorded to the nearest millimeter using a University of North Carolina periodontal probe-15 (UNC-15, GDC Company, India). Sites with deepest pocket depth were selected for sample collection.

Microbiological Examination

Sample Collection

Total 15 subgingival plaque samples were collected from each group (total subgingival plaque samples collected: $2 \times 15 = 30$) using sterile Gracey curettes #1/2, #5/6, #7/8, #15/16 from the deepest selected pocket after the removal of supragingival plaque. Immediately samples were placed in polypropylene tubes (of 1.5 mL) containing 50 µL TE [10 mm tris-HCl, 1 mm ethylenediaminetetraacetic acid (EDTA), pH 7.6] and stored at −80°C until molecular processing was done.[6]

Nucleic Acid Extraction

For nucleic acid extraction, mechanical and thermal methods were used. The subgingival plaque samples were directly suspended in 50 µL TE buffer (10 mm tris-HCl, 0.1 mm EDTA, pH 7.6). The suspensions were incubated at 56°C for 30 minutes and then at 100°C for 8 minutes. After incubation, the suspension was centrifuged and 2 µL aliquots of the resulting supernatants were used as templates for polymerase chain reaction (PCR).

General Conditions for PCR

Amplification and detection of DNA by PCR were performed on a LightCycler 2.0 (Roche Applied Science, Germany). A primer pair specific (Kulik et al. 2001)[7] for 16S rRNA gene of Euryarchaea (specific for *Methanobrevibacter (M.) oralis*) was used (300fEyAr 5′-GC (A/G) (A/G) GAGCCCGGAGATGG-3′ and 954rEyAr 5′-CGGCGTTGA (A/G) TCCZZTTAAAC-3′) (Bioserve biotechnologies Ltd., Hyderabad, India). The PCR was performed in a total volume of 50 µL, containing 1 µL of template DNA, 2 mM of $MgCl_2$, 25 pmol of each primer, 0.2 mM of deoxynucleoside triphosphates, and 2U of Platinums Taq DNA polymerase. Amplification was performed with the PCR system (Applied Biosystems). Steps involved were 35 cycles of 94°C (30 seconds), 60°C (30 seconds), and 72°C (40 seconds) followed by a 7-minutes extension at 72°C. This was done on an automated cycler.

Aliquots of 5 µL of each PCR product were subjected to electrophoresis on 1.5% agarose gel. The gel was stained with ethidium bromide (1 mg μL^{-1}) and assessed under UV light.

Statistical Analysis

Significant differences in frequency of archaea-positive subjects between disease types were assessed by the Chi-square test. The Mann–Whitney *U* test was used to assess differences between the groups. The Pearson correlation test was used to find the correlation between archaea-positive subjects with clinical parameters.

Results

The distribution of samples along with respective mean and standard deviation (mean ± SD) values of PPD, CAL, and prevalence of *M. oralis* of groups I and II are depicted in Table 1.

The prevalence of *M. oralis* detected in group I was 1, which accounts for 6.7%, and in group II it was 6, which accounts for 40% prevalence. On statistical analysis, when the prevalence was compared between the groups, i.e., group I compared to group II, there was significant prevalence ($p = 0.04$) as depicted in Table 2.

The analysis of the clinical features showed that when the comparison was made between groups I and II, it showed statistically highly significance ($p = 0.001$) for PPD and CAL as shown in Table 1. No correlation was established between the prevalence of group I with all the clinical parameters ($p > 0.05$), but in group II the clinical parameters PPD ($p = 0.051$) and CAL ($p = 0.049$) were significantly correlated to the prevalence of *M. oralis* as in Table 3.

Table 1: Descriptive statistics of clinical and microbiological parameters

	Group I	*Group II*	*Group I vs group II*	
Clinical parameter (mean ± standard deviation)			*Z value*	*p value*
PPD (mm)	3.77 ± 0.7	8.93 ± 1	−4.734	0.001
CAL (mm)	0.33 ± 0.4	5.13 ± 1	−4.788	0.001
Prevalence of M. oralis (in %)			*Post hoc test (Mann–Whitney U test)*	
Present	6.7	40		
Absent	93.3	60		

Group I: Periodontally healthy group; Group II: Chronic periodontitis group. PPD, probing pocket depth; CAL, clinical attachment level. *p* value <0.001—highly significant using post hoc test (Mann -Whitney *U* test)

Table 2: Inter (between the) group comparison of microbial parameters

	Groups			
	I	*II*	*Total*	*p value*
Prevalence of *M. oralis*				
Present	1	6	7	0.04
Absent	14	9	23	

Group I: Periodontally healthy group; Group II: Chronic periodontitis group. *p* value <0.05—significant, using Chi-square test

Table 3: Correlation between the clinical parameters and prevalence of *M. oralis* among the groups

	p value (two-tailed)	
Clinical parameters	*Group I*	*Group II*
PPD	0.297	0.051
CAL	0.165	0.049

Group I: Periodontally healthy group; Group II: Chronic periodontitis group. PPD, probing pocket depth; CAL, clinical attachment level. *p* value: Correlation is significant at <0.05 level, using Pearson correlation

Discussion

Recent scientific progress in the identification of microbes by molecular technologies has gained its momentum in the field of periodontology. More importantly, noncultivable organisms that were not able to be identified with traditional culture methods are now identified and detected using PCR, such as bacteria, viruses, etc. This new PCR molecular technology has shown greater interest in identifying new organisms in the oral cavity as well as periodontal diseases (*A. naeslundii*, *S. sputigena*, *R. dentocariosa*, *E. corrodens*, and clone I025 from the *TM7* phylum).[8] Recently, archaea have gained their attention in the field of periodontal research.

The archaea are unique in the sense that they are omnipresent right from most extreme ecological conditions to the gut and oral cavity of humans.[9] Recently, few studies have also reported presence of these organisms in oral abscess, tongue coating, and endodontic lesions.[10,11] Studies have shown the prevalence of *M. oralis* in the subgingival plaque samples of periodontal diseases (Lepp et al. 2004, Li et al. 2004, Yambae et al. 2008).[9,10,12]

The present study is a randomized single-blind study design. In our study, prevalence of *M. oralis* in subgingival plaque samples of chronic periodontitis was assessed along with clinical parameters and correlation was made between the microbial archaea and clinical parameters.

The clinical comparison of PPD and CAL between groups I and II showed statistically significance ($p < 0.01$), which is consistent with studies by Ashok et al. (2013)[13] and Yamabe et al. (2008);[12] study done by Amelie et al. (2013)[14] is in accordance with the CAL levels in groups I and II.

The result showed the prevalence of *M. oralis* that was statistically significant ($p < 0.05$) when groups I and II were compared with not much difference statistically. This may have been due to the small number of subjects included in the study. The results of group I of our study are in accordance with Ashok et al. and Amelie et al., and not in accordance with Yamabe et al. and Lepp et al. Group II results are not consistent with the study done by Lepp et al. (2004), which showed much higher prevalence in chronic periodontitis. The correlation with group II for mean PPD and CAL shows clinical significance with prevalence of *M. oralis*. Results found with group II are in accordance with study by Lepp et al.[10] and study by Ashok et al.[13]

M. oralis may suggest its positive association with the subgingival environment of the periodontal pockets. The present study showed high prevalence of *M. oralis* in the chronic periodontitis group and not in the healthy group.

The anaerobic archaea use H_2 and NH_4, which are the substrates produced by other microbes for the methane production.[15] Studies have shown in humans that removal of H_2 by archaea forms a synanthropic relationship with local bacteria and improves its fermentation efficiency, which favors its survival. This kind of relationship between microbes may increase the microbial ability in generating energy from nondigestible complex food, thus increasing the survival chances of the anaerobic microbes.[15]

Our study has limitations/drawbacks like less sample size, study examination was done by one examiner, DNA of *M. oralis* was extracted by means of thermomechanical cell lysis using both heat and pressure, which may explain differences in the prevalence of methanogenic archaea[16] and also during processing in PCR which was not standardized; all these may have affected the outcome of the data and results.

This study shows prevalence and quantification of archaea in the subgingival ecological niche of chronic periodontitis patients, which may indicate archaea as an active component and that it may be associated with periodontal disease.

Conclusion

The study detects the presence of archaea in subgingival microbial niche of chronic periodontitis patients. Future research may be considered with large clinical sample size, longer evaluation period, and a specific primers/standardized protocol for the PCR detection method to evaluate presence of archaea in disease sites.

Clinical Significance

This study is an initial attempt to understand the prevalence of archaea in periodontal disease and health. This gives us an insight into how diverse is our oral cavity in terms of microbiology. Hence, one has to determine the exact mechanism of action of the disease causing organism and form a strategist treatment plan. archaea are still unknown to the oral cavity and this study indicates their importance in periodontal therapy.

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