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Short Communication

Microbiology Section

Metagenomic Characterisation of Microorganisms in the Dental Plaque- A Pilot Study

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ABSTRACT

Introduction: Progression of dental plaque gives rise to periodontal disease. The oral cavity has a diverse flora of microorganisms and caries is often caused by members of the oral microbiome. Metagenomic techniques and next-generation sequencing technology have been used to analyse microbiomes.

Aim: To analyse the components of the dental plaque by culture and metagenomics techniques.

Materials and Methods: The present study was a pilot study in which analysis of the components of the dental plaque by bacteriological culture, 16s ribosomal Ribonucleic acid (rRNA) Polymerase Chain Reaction (PCR) with region specific primers and sequencing of V3-V4 regions, using the Illumina platform was performed.

Results: Ten different phyla were identified (Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Actinobacteria, TM7, Spirochaetes, Tenericutes, SR1, GN02). Bacteriodetes was the most abundant phylum in the plaque samples. Firmicutes was less abundant in the plaque samples, when compared to the control. *Streptococci* were more abundant in the control (57.93%) when compared to plaque samples (10.7%, 19.3%). Routine culture grew only Viridans group *streptococci* (VGS).

Conclusion: The 16S rRNA gene sequencing is a useful method for studying the bacterial composition of the dental plaque.

Keywords: Bacterial composition, Oral microbiome, Sequencing

INTRODUCTION

The dental plaque is a mass of bacteria that grows on the surface of tooth, as a sticky colourless deposit. Progression of dental plaque can give rise to caries and periodontal disease. Oral bacteria can colonise and cause disease in different sites in the oral cavity because of specific adherence mechanisms. The oral cavity has a diverse flora of microorganisms which is considered to be very complex and second only to the gut microbiome [1]. A large proportion of oral bacteria cannot be cultured, and therefore, traditional microbiological techniques give an incomplete picture of the natural communities inhabiting the dental plaque. The VGS occur as normal flora in the oral cavity and belong to the Phylum Firmicutes. Certain species of VGS are associated with dental caries [2]. The different sites in the oral cavity are inhabited with different microbes which are specific for the site. Microbes from all the sites of the oral cavity together make up the salivary microbiome [3]. The dental plaque is defined as "the microbial community that develops as a structurally- and functionally-organised biofilm on the tooth surface, embedded in a matrix of polymers of bacterial and host salivary origin" [4]. The development of metagenomic techniques and next-generation sequencing technology allows the study of whole bacterial communities by analysing the total Deoxyribonucleic Acid (DNA) pool from complex microbial samples [5].

Therefore, aim of the present study was to identify the bacteria in plaque samples by culture and metagenomic techniques.

MATERIALS AND METHODS

The present study was a pilot study conducted in outpatient dental clinic at Ragas Dental College, Chennai, Tamil Nadu, India and samples were collected over a period of one week in May 2017.

Inclusion criteria: Plaque samples were collected from 10 patients (five males, five females) aged 18 to 60 years in the morning after brushing of teeth and before breakfast from patients who were in good health, without any history of recent infections were included in the study.

Exclusion criteria: The exclusion criteria were antibiotic treatment or any dental treatment/procedures such as restorations, root canal therapy, fluoride application or oral prophylaxis in the preceding three months. Patients who were smokers, alcoholics, pregnant and lactating women, and patients on long-term anti-inflammatory/immunosuppressive therapy, were also excluded from the study.

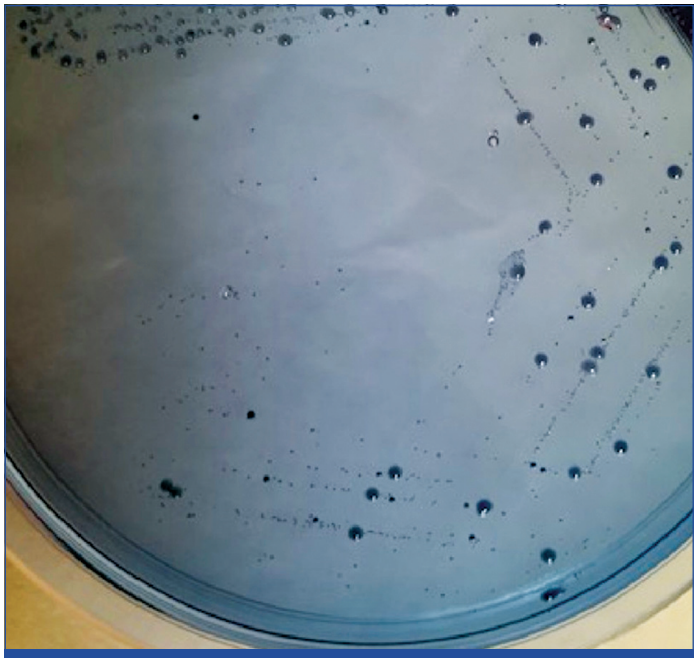
Clinical examination did not reveal periodontitis or gingivitis in any of the study subjects, who had come for routine scaling/periodic dental check-up. All procedures were carried out in accordance with ethical standards and informed consent was obtained from the patients. The patients were examined (entire dentition) for the presence of plaques at four sites per tooth, namely the mesial, buccal, lingual and facial surfaces. Generally plaque was collected from all the teeth, wherever present, but they were predominantly on the labial surfaces of the upper anteriors (11,12,13,21,22,23) and lower anteriors (31,32,33,41,42,43); and the lingual surfaces of the lower posteriors (36,37,46,47). The plaque was collected by gently scraping with a sterile curette along the surface of the tooth (buccal and/or lingual) in one single stroke, and stored in Phosphate Buffered Saline (PBS) at -20°C, for further analysis. Care was taken not to induce bleeding from the sites. The plaque index was calculated using the O'Leary index [6] with the following formula:

$$\frac{\text{No. of sites with plaque}}{\text{No. of sites evaluated}} \times 100$$

The plaque samples were mixed with 1 mL PBS vortexed for few seconds and inoculated on Mitis Salivarius Agar (MSA) and 5% sheep blood agar. An oral rinse in 1 mL of sterilised PBS was collected from an individual with good oral health was included as the control and processed in the same manner. The plates were incubated at 37°C in 5-10% of CO_2 for 24 hours, and preliminary identification of the isolates was done based on colony morphology. An aliquot of each sample was stored at +4°C for metagenomic analysis.

The isolates were gram stained and pure cultures of different morphotypes [Table/Fig-1] were maintained in Brain Heart Infusion

(BHI) broth. The phenotypic characterisation was performed by using conventional biochemical tests which includes Voges-Proskauer test, fermentation of mannitol, fermentation of sorbitol, hydrolysis of esculin and arginine and urease test [7].



[Table/Fig-1]: Viridans group *Streptococci* on Mitis-Salivarius agar. *S.mitis* appears as pin point blue colonies and *S.salivarius* appears as large "gum drop" type blue colonies.

Metagenomic study was done on two representative plaque samples (PQ1 and PQ2) and the control sample (oral rinse collected from healthy individual). DNA was extracted, purity and concentration were estimated and libraries were prepared, as described previously [8]. The amplification of V3–V4 region of 16S gene was done using Illumina platform (Genotypic Technology Pvt., Ltd., Bangalore, India) using region specific primers [9]. The amplified 16S rRNA was sequenced and analysed using Quantitative Insights Into Microbial (QIIME). The organisms were classified using a database of 16S rRNA data based on the Greengenes database (http://greengenes.lbl.gov/).

RESULTS

The plaque index of the patients in the study group ranged from 38-67% [Table/Fig-2]. Routine bacteriological culture showed a mixture of microorganisms including VGS which was more abundant in the control when compared to the plaque samples. *Streptococcus mitis* and *Streptococcus salivarius* was present in all the samples, but colony counts were much higher in the control sample. Eighteen strains of VGS were isolated from the 10 plaque samples and *Streptococcus salivarius* was the predominant isolate (11/18). Metagenomic analysis showed 10 different phyla such as Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Actinobacteria, TM7, Spirochaetes, Tenericutes, SR1 and GN02. Bacteroidetes, TM7, Spirochaetes, and Fusobacteria were the predominant phyla in the plaque samples, whereas, Firmicutes was predominant in the control. Bacteriodetes was the most abundant phylum in the plaque samples (33.13%; 23.38%) and least abundant (1.6%) in the control [Table/Fig-3]. Tenericutes and Synergistetes were abundant in one plaque sample but less abundant in the other. The families Streptococcaceae, Gemellaceae, and Micrococceae were most common in the control, whereas, Peptostreptococcaceae, Prevotellaceae, Porphyromonadaceae, Campylobacteriaceae, Fusobacteriaceae, Planococcaeae, Leptotriciaceae, and Lachnospiraceae, were more common in the plaque samples.

Streptococcus was more abundant in the control (57.93%) when compared to the plaque samples (19.3%; 10.7%). *Capnocytophaga*, *Porphyromonas* and *Prevotella* were the most abundant genera in the plaque samples [Table/Fig-4].

Patient No.	Age	Sex	Plaque index (%)	Viridans Group *Streptococci*
1	32	F	45	*mitis, salivarius, sanguinis*
2	18	F	48	*sanguinis; mitis*
3	38	M	53	*salivarius; mitis*
4	36	M	67	*salivarius; mitis*
5*	30	M	59	*mitis; salivarius*
6	42	F	52	*mitis*
7*	25	F	43	*mitis; salivarius*
8	60	F	51	*salivarius; mitis*
9	23	M	38	*mitis; salivarius*
10	27	M	54	*salivarius*

[Table/Fig-2]: Patient details with plaque index.
*Processed further for metagenomics study

Phylum	PQ1* (%)	PQ2 * (%)	Control (%)
Firmicutes	40.09	23.14	65.54
Bacteriodetes	33.13	23.38	1.6
Proteobacteria	6.14	27.18	19.45
Fusobacteria	7.89	8.61	3.25
Actinobacteria	4.83	11.56	10
TM7	5.52	3.49	0.089
Spirochaetes	0.708	0.549	0.007
Tenericutes	0.692	0.01	0.005
SR1	0.0003	0.7734	0.005
GN02	0.0003	1.11	0.01

[Table/Fig-3]: Distribution of phyla in plaque and control samples.
*PQ1 and PQ2- plaque samples

Genus	PQ1 *(%)	PQ2 *(%)	Control (%)
Streptococcus	19.3105	10.7607	57.9338
Capnocytophaga	17.2155	7.5416	0.3098
Porphyromonas	7.72	8.4952	0.7051
Prevotella	4.5772	2.7844	0.243
Leptotrichia	4.4725	4.5851	2.7786
Veillonella	3.2366	1.7325	1.3078
Neisseria	2.6612	10.2147	14.5384
Fusobacterium	1.9953	4.0151	0.4799
Actinomyces	1.934	2.1859	5.9827
Gemella	1.2894	0.9541	5.1103
Rothia	1.1306	0.943	3.0519

[Table/Fig-4]: Distribution of genera in plaque and control samples.
*PQ1 and PQ2- plaque samples

DISCUSSION

The dental plaque consists of a diverse mixture of bacteria which varies depending on anatomic factors, the salivary flow and the local oral environment including the pH [10]. Some bacteria produce acid which cause solubilisation of the tooth enamel, leading to initiation of dental decay. Bacteria such as *S. mutans*, lactobacilli, and *Actinomyces naeslundii* are implicated in the initiation and progression of caries. The aetiology of caries, however is complex and aciduric bacteria such as *S. anginosus* and *S. oralis* have been isolated in subjects without caries; and bacteria without aciduric properties have been isolated from caries teeth [11]. *S. mutans* has been associated with dental caries [12,13]; however, at times, they are not detected at sites of decay [14]. Species of the genera *Lactobacillus, Veillonella, Propionibacterium, Bifidobacterium* and non mutans *Streptococci* have been identified in plaque samples [15]. Present study found that organisms such as *Capnocytophaga* and non mutans *Streptococci* are important constituents of the plaque microflora and did not detect *S. mutans* or Lactobacilli in any of our samples. There have been conflicting opinions on the association of *S. mutans* with dental

caries. *S. mutans* has been isolated from tooth surfaces with sound enamel and hence, it appears that organisms other than *S. mutans* could contribute to tooth decay [16].

A large proportion of the microbial flora is non cultivable; and routine culture detected only VGS (phylum Firmicutes). By using metagenomic techniques, nine additional phyla were identified. Understanding the complexities of the plaque microbiome and its varied microbial community is important in the management of dental caries. Characteristics of the microflora of the plaque determine its cariogenicity; detection of caries causing bacteria in abundance would help to predict cariogenicity in an individual and initiate prophylactic measures.

Limitation(s)

The sample size in the present study was too small to arrive at a clear conclusion; further metagenomics analysis which was expensive could be performed only on two samples. However, as a pilot study, it provides sufficient data to plan a larger study of the oral microbiome to investigate cariogenicity and its relation to oral health.

CONCLUSION(S)

Though *S. mutans* is known to be associated with dental caries, present study found that organisms such as *Capnocytophaga* and non mutans *Streptococci* are important constituents of the dental plaque. Present study did not detect *S. mutans* or Lactobacilli in any of the samples. Present study findings, reiterate the fact that caries is polymicrobial in nature and considering *S. mutans* alone as a "therapeutic target" may not help in the prevention of dental caries. The use of bacterial cultures to study the constituents of dental plaque has its limitations. Detailed characterisation of the plaque microflora is possible by the use of metagenomic techniques and sequencing of the 16S rRNA gene. Further studies are recommended on high level dental plaque in larger group of patients, to see the effectiveness of gene sequencing.

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Contributions of authors: Dr. AVB: Selection of participants, collection of samples and patient care, approving final draft of manuscript; Ms ADJ: Processing of samples for bacteriological culture, identifying bacterial aetiology, approving final draft of manuscript; Dr. TM: Conceptualisation and planning of the study, analysing metagenomic data, manuscript writing.

REFERENCES

[1] Deo PN, Deshmukh R. Oral microbiome: Unveiling the fundamentals. J Oral Maxillofac Pathol. 2019;23(1):122-28. Doi: 10.4103/jomfp.JOMFP_304_18.
[2] de Soet JJ, Nyvad B, Killian M. Strain-related acid production by oral streptococci. Caries Res. 2000;34(6):486-90.
[3] Krishnan K, Chen T, Paster BJ. A practical guide to the oral microbiome and its relation to health and disease. Oral Dis. 2017;23 (3):276-86.
[4] Marsh P, Martin M. Oral Microbiology. 6th ed. Edinburgh: New York; Elsevier, 2016, ISBN: 9780702061066, eBook ISBN: 9780702061745.
[5] Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. J Clin Microbiol. 2007;45(9):2761-64.
[6] O'Leary TJ, Drake RB, Naylor JE. The plaque control record. J Periodontol. 1972;43(1):38. Doi: 10.1902/jop.1972.43.1.38. PMID: 4500182.
[7] Facklam R. What happened to the streptococci: Overview of taxonomic and nomenclature changes. Clin Microbiol Rev. 2002;15(4):613-30.
[8] Menon T, Kalyanaraman S, Srinivasan S. Composition of the oral microbiome in patients with coronary artery disease. J Clin of Diagn Res. 2020;14(9):DM01-DM03. https://www.doi.org/10.7860/JCDR/2020/45246/14034.
[9] Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41(1):e110.1093/nar/gks80822933715.
[10] Donoghue HD. Composition of dental plaque obtained from eight sites in the mouth of a ten year old girl. J Dent Res. 1974;53(5):1289-93.
[11] Brailsford SR, Shah B, Simons D, Gilbert S, Clark D, Ines I, et al. The predominant aciduric microflora of root-caries lesions. J Dent Res. 2001;80(9):1828-33. Doi: 10.1177/00220345010800091101.
[12] Loesche WJ, Rowan J, Straffon LH, Loos PJ. Association of *Streptococcus mutans* with human dental decay. Infect Immun. 1975;11(6):1252-60.
[13] Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiol Mol Biol Rev. 1986;50(4):353-80.
[14] Banas JA, Drake DR. Are the mutans streptococci still considered relevant to understanding the microbial etiology of dental caries? BMC Oral Health. 2018;18(1):129.
[15] Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol. 2008;46(4):1407-17.
[16] Kleinberg I. A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: An alternative to *Streptococcus mutans* and the specific-plaque hypothesis. Crit Rev Oral Biol Med. 2002;13(2):108-25.

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