DOI: 10.7860/JCDR/2020/45246.14034

Short Communication

Microbiology Section

Composition of the Oral Microbiome in Patients with Coronary Artery Disease

THANGAM MENON[1], SUPRAJA KALYANARAMAN[2], SEETHALAKSHMI SRINIVASAN[3]

ABSTRACT

Introduction: Distinct microbial communities reside in the oral cavity and the composition of the oral microbiota has important implications for human health and disease. Identification of bacterial flora of the microbiome is done by metagenomic analysis of 16S ribosomal RNA sequences.

Aim: The aim of this study was to characterise the human microbiome in patients with Coronary Artery Disease (CAD) in comparison with the normal human microbiome.

Materials and Methods: A pilot study was carried out in tertiary hospital, Chennai. Oral mouthwash samples collected from nine patients with CAD were selected, with one control group. They were studied by metagenomic analysis of V3-V4 region of 16SrRNA gene sequences. Sequencing of the variable V3 and V4 regions was done using Illumina platform.

Results: The six major phyla, *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes*, and *Fusobacteria* contained 99% of the taxa in all the samples analysed.

Conclusion: Diversity of the microbiome in patients with CAD was similar to the normal human microbiome.

Keywords: Dysbiosis, Metagenomic analysis, Phylum, Taxa

INTRODUCTION

The ecological community of microorganisms living within the human host is referred to as microbiota and is a mixture of symbiotic, commensal and pathogenic microorganisms. Oral microbiome is defined as the collective genome of microorganisms that reside in the oral cavity. Dysbiosis of the oral microbiota has been linked to several diseases in human beings [1-3]. Some of the oral bacteria are known to be capable of triggering inflammatory processes leading to initiation and progression of Cardiovascular Disease (CVD) indicating the existence of a dynamic relationship between inflammation and the oral microbiome. Oral microbiota has been reported to contribute to the development of atherosclerosis [4].

Oral health promotion has resulted in improvements in periodontal health, and modifications of systemic inflammatory markers [5]. Many of the members of the microbiota are fastidious or cannot be cultured and the advent of culture-independent methods has greatly improved their detection. The diversity and composition of the microbial communities in the oral cavity is studied by metagenomic profiling of the collective genomes of whole microbial communities and modern molecular techniques such as high-throughput sequencing [6]. The 16S rRNA gene which is present in all prokaryotes is approximately 1600 base pairs long and includes nine hypervariable regions (V1-V9). Partial sequences of the gene targeting the hypervariable regions are used for bacterial identification. In recent years, analysis of 16S ribosomal RNA sequences have been used for the taxonomic identification of bacterial strains [7]. There are very few studies on CAD patients, particularly from India. This study reported the comparison of the oral microbiome in patients with CAD and healthy individuals by metagenomic analysis of V3-V4 region of the 16SrRNA gene sequences.

MATERIALS AND METHODS

A cross-sectional pilot study was carried out between November 2018 and July 2019, in a tertiary hospital specialising in cardiac care in Chennai city, Tamil Nadu, India. The study was approved by the Institutional Ethics Committee (EC Reg no: ECR/140/Inst/TN/2013/RR-16). Subjects of the study were nine adults with coronary angiographic characteristics consistent with significant CAD. Informed consent was obtained from all the patients. Patients admitted for acute myocardial infarction, arrhythmias, cardiac failure or treatment of cardiomyopathies were excluded from the study. One healthy subject with no history of cardiac disease was included as control.

Oral mouthwash samples in phosphate buffered saline were collected in sterile containers. Culture was done on blood agar, MacConkey agar and Mitis salivarius agar. Plates were incubated at 37°C for 24-48 hours after which colonies were identified by standard procedures [8]. Samples were stored at +4° C for metagenomics analysis. DNA was extracted and quantitated. PCR was done using region specific primers to amplify V3–V4 region of 16S rRNA gene [9]. The primers used were:

Forward primer: 5'- CCTACGGGGNGGCWGCAG- 3'

Reverse primer: 5'-GACTACHVGGGTATCTAATCC-3'

Illumina adapter overhang nucleotide sequences were added to the gene specific sequences. The amplicon size was 465 bp. PCR amplicons were analysed on 1.2% agarose gel. Further the libraries were normalised and pooled for sequencing. Sequencing of the variable V3 and V4 regions of the 16S rRNA gene was done using Illumina platform (Genotypic Technology Pvt., Ltd., Bangalore, India).

STATISTICAL ANALYSIS

The paired end V3-V4 reads were checked using FastQC [10] and joined using Fastq-join. Analysis was done using Quantitative Insight into Microbial Ecology (QIIME) [11]. QIIME uses the Ribosomal Database Project (RDP) classifier to assign taxonomic data to each representative sequence using a cut-off of ≥97% sequence similarity against the reference database. Relative abundance was calculated using the R package Non-negative Matrix Factorization (NMF).

RESULTS

The nine patients (7 males, 2 females) aged 45-76 years and were included from the tertiary hospital patients. The clinical and demographic details of the patients are given in [Table/Fig-1]. All specimens grew abundant normal flora, predominantly Viridans Group Streptococci (VGS). Among the VGS, *Streptococcus salivarius* and *Streptococcus mitis* were most frequently isolated in patients while the control sample grew *S. mitis*. When the V3-V4 region of 16S rRNA gene were analysed, it was seen that *Firmicutes* and *Bacteroidetes* were the predominant phyla and accounted for more than 60% of the taxa in all the patients. *Proteobacteria* was the predominant phylum in the control and together with *Firmicutes* accounted for more than 60% of the taxa. Eighteen bacterial phyla were identified within the combined dataset [Table/Fig-2].

S. No.	Sample ID	Age	Sex	Height (cm)	Weight (kg)	Clinical history	Diabetic status	Lipid profile	Culture results
1	CAD 11	61	Female	158	56	TVD-s/p OPCAB CAG done on 21/11/18	Diabetic	Not available	Abundant *S.mitis*, occasional *S. salivarius*
2	CAD 12	64	Male	162	61.3	TVD-CAG done on 16/11/18	Diabetic	TC-96, TGL-121, HDL-32, LDL -62	Gram negative diplococci, *S. mitis, S. salivarius, S.mutans*
3	CAD 13	63	Male	150	65	SVD CAG done on 5/12/18	Diabetic	TC-100, TGL-188, HDL-30, LDL-66	Gram negative diplococci, abundant *S.mitis*, few *S. salivarius*, very few *S. mutans*, occasional Enterococci
4	CAD 14	65	Male	160	87.5	TVD-CAG done on 1/12/18	Diabetic	TC-197, TGL-189, HDL-33, LDL-136	Abundant *S.mitis*, *S.epidermidis*
5	CAD 15	53	Female	161	68	SVD, PTCA-stent on 5/12/18	Diabetic	TC-116, TGL-107, HDL-35, LDL-75	Abundant *S.mitis*, scanty Enterococci
6	CAD 17	76	Male	160	51.7	TVD, CAG done on 30/11/18	Diabetic	TC -173, TGL -163, HDL -24, LDL-143	Micrococci, gram negative diplococci, *S. mitis*, occasional Enterococci
7	CAD 18	51	Male	172	63	TVD, CAG done on 12/11/18	Diabetic	TC-111, TGL-173, HDL-34, LDL-67	Abundant *S. mitis*, *S. salivarius*
8	CAD 20	46	Male	166	78.5	TVD- CAG done on 14/11/18	Diabetic	TC-137, TGL- 223, HDL-30, LDL-89	*S. mitis*, *S. salivarius*, few Enterococci.
9	CAD 21	49	Male	160	60	TVD-s/p OPCAB	Diabetic	TC- 162, TGL-166, HDL-27, LDL-117	*S. salivarius*

[Table/Fig-1]: Demographic details and clinical history of patients.
CAD: Coronary artery disease; TVD: Triple vessel disease; SVD: Single vessel disease; CAG: Coronary angiography; s/p: Status post-surgery; PTCA: Percutaneous transluminal coronary angioplasty; OPCAB: Off pump coronary artery bypass; TC: Total cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; TGL: Triglycerides

Patient ID	CAD11	CAD12	CAD13	CAD14	CAD15	CAD17	CAD18	CAD20	CAD21	Control
Clinical findings	TVD	TVD	SVD	TVD	SVD	TVD	TVD	TVD	TVD	
Age/Sex	61/F	64/M	63/M	65/M	53/F	76/M	51/M	46/M	49/M	32/F
Phylum										
Firmicutes	45.9381	53.2765	34.6035	75.4709	37.643	69.996	64.8542	66.9725	70.9569	30.9947
Bacteroidetes	26.5174	17.7554	28.6291	7.7481	24.5752	13.5547	16.5183	10.1216	16.8357	19.158
Actinobacteria	8.9271	6.2999	2.296	1.0211	5.4395	0.9515	10.1754	6.7335	7.5231	3.7984
Proteobacteria	8.2379	11.2332	13.8162	12.172	22.0904	10.3896	5.4761	15.9481	2.2843	34.9574
Fusobacteria	7.3987	10.3626	9.9435	3.1587	9.6968	3.9489	2.2326	0.1787	1.9498	9.3998
Spirochaetes	2.1427	0.0864	6.6048	0.2048	0.0442	0.7344	0.1762	0.0187	0.0421	0.996
Tenericutes	0.4046	0.0638	1.4877	0.0098	0.0031	0.1742	0.009	0.0021	0.0444	0.0371
TM7	0.2397	0.7389	1.1436	0.1711	0.3859	0.0884	0.5003	0.0083	0.2187	0.5245
Synergistetes	0.1568	0.07	1.3571	0.0084	0.0244	0.1274	0.0566	0.0021	0.1158	0.0106
Planctomycetes	0.0288	0.0412	0.0044	0.0084	0.0031	0.013	0	0.0021	0.0152	0.0353
Verrucomicrobia	0.0046	0	0	0	0	0	0	0	0	0.0018
Thermi	0.0023	0	0	0	0	0	0	0.0125	0.014	0
Cyanobacteria	0.0012	0.0041	0	0	0	0	0	0	0	0
SR1	0	0.0123	0.0756	0.0266	0.093	0	0.0013	0	0	0.0406
GN02	0	0	0	0	0.0015	0	0	0	0	0
Chloroflexi	0	0.0268	0.0267	0	0	0.0182	0	0	0	0
Chlorobi	0	0	0.0104	0	0	0	0	0	0	0
Acidobacteria	0	0.0288	0.0015	0	0	0.0039	0	0	0	0.0459

[Table/Fig-2]: Distribution of phyla.
TVD: Triple vessel disease; SVD: Single vessel disease

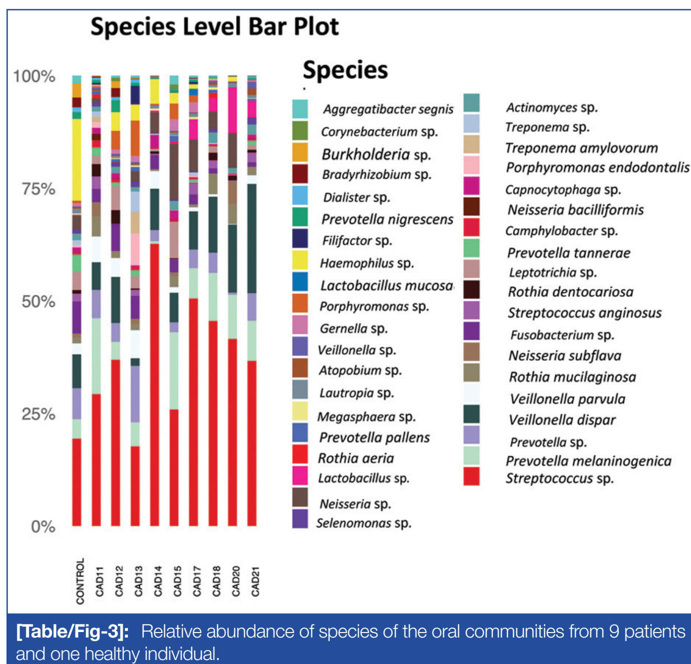
In 8/9 patient samples and in the control sample, the six major phyla, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes*, and *Fusobacteria*, contained 99% of the taxa and the remaining phyla, *Tenericutes, TM7, Synergistetes, Planctomycetes, Verrucomicrobia*, *SR1*, *Chloroflexi*, etc., contained less than 1% of the taxa. Phyla such as *Verrucomicrobia* which was present in the control was very rare in CAD patients (1/9). *Cyanobacteria* and *Chlorobi* were absent in the control and rare in CAD patients.

In the single control sample, *Proteobacteria* (34.9%), *Firmicutes* (31.99%), and *Bacteroidetes* (19.1%) were the most abundant. In the patients, *Firmicutes* varied between 35% and 75%, *Proteobacteria*, from 2.2% to 22.0%, and *Bacteroidetes* from 7.7% to 28.6%. The most common genus was *Streptococcus* in all the samples, followed by *Prevotella* and *Veillonella* [Table/Fig-3].

DISCUSSION

Previous studies on the composition of the oral microbiota in healthy individuals and in patients with atherosclerosis have shown that the oral microbiota of patients is dominated by *Firmicutes* (69%) followed by *Bacteroidetes* (10%), *Actinobacteria* (9%), *Fusobacteria* (6%), *Proteobacteria* (5%), and <1% of *Spirochaetes*, *TM7*, *SR1*, and *Tenericutes*. The microbiome in healthy individuals was found to be similar except for more representation from *Firmicutes* (76%) and less of *Bacteroidetes* (6%) and *Fusobacteria* (3%) [12]. Other studies of the oral microbiome in healthy individuals have shown that *Firmicutes* though most abundant accounted for only 33.2% of all sequences, *Proteobacteria* for 27.5%, *Bacteroidetes* for 16.6% and *Actinobacteria* for 14.5% of all sequences [13].

In the present study, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were the most abundant phyla in all the patient samples with large



[Table/Fig-3]: Relative abundance of species of the oral communities from 9 patients and one healthy individual.

inter-sample variations. The phylum *Proteobacteria* was more abundant in the control sample than in any of the patient samples. Though only one control sample was analysed in this study, the abundance of *Firmicutes*, *Proteobacteria* and *Bacteroidetes* was comparable to a study done by Bik EM et al., on 10 healthy individuals [13]. It was also comparable to author's earlier study which investigated the oral microbiome in four healthy individuals, according to which *Bacteroidetes*, *Proteobacteria* and *Firmicutes* were the predominant phyla seen in oral cavity of healthy individuals. There was considerable diversity in the microbiota and uncultured bacteria showed the highest abundance [14]. In the present study *Fusobacteria* ranged between 0.1% to 10.3% in patients and 9.3% in the control. It appeared that the abundance of *Fusobacteria* varied widely in the patient population. Other studies have reported 6% in patients [12] and 3%-6.7% in controls [12,13]. The abundance of *Fusobacterium* is said to positively correlate with levels of LDL cholesterol [12], but no such association was seen in the present study. *Streptococcus* spp., and *Veillonella* spp., were abundant in the patient samples which was comparable to a previous study [15]. Both organisms have previously been isolated from atherosclerotic plaques and plaque microbiota are said to be derived from the oral cavity and/or the gut.

Limitation(s)

This was a pilot study and the sample size of this study was small. The high cost of sequencing is restrictive and pilot studies help to determine statistical power and optimum sample size [16]. Metagenomics research generates large number of genome sequences and consumes heavy computing resources and time. Sample sizes are kept to an optimum, particularly in low resource settings to avoid redundancy and reduce costs.

CONCLUSION(S)

Advances in molecular biology and bioinformatics have made microbiome research possible. A 16SrRNA gene sequencing is superior to culture methods for studying abundance and diversity of the oral microbiome. Microbial communities in the oral cavity varied considerably between individuals and there was no overall difference when the oral microbiome in CAD patients was compared to the normal oral microbiome.

Acknowledgement

The financial support provided by University Grants Commission, New Delhi is gratefully acknowledged.

REFERENCES

[1] Miele L, Giorgio V, Alberelli MA, De Candia E, Gasbarrini A, Grieco A. Impact of gut microbiota on obesity, diabetes, and cardiovascular disease risk. Curr Cardiol Rep. 2015;17:120.
[2] Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med. 2013;19:576-85.
[3] Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, et al. Commensal microbiota affects ischemic stroke outcome by regulating intestinal gamma delta T cells. Nat Med. 2016;22:516-23.
[4] Fåk F, Tremaroli V, Bergström G, Bäckhed F. Oral microbiota in patients with atherosclerosis. Atherosclerosis. 2015;243(2):573-78.
[5] Lam OLT, Zhang W, Samaranayake LP, Li LSW, McGrath C. A systematic review of the effectiveness of oral health promotion activities among patients with cardiovascular disease. Int J Cardiol. 2011;151(3):261-67.
[6] Kilian M, Chapple I, Hannig M, Marsh PD, Meuric V, Pedersen AML, et al. The oral microbiome- An update for oral healthcare professionals. Br Dent J. 2016;221:657-66. https://doi.org/10.1038/sj.bdj.2016.865.
[7] 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. DOI: 10.1128/JCM.01228-07.
[8] Chapman GH. The isolation of streptococci from mixed cultures. J Bacteriol. 1944;48(1):113-14.
[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):e1. DOI:10.1093/nar/gks808.
[10] Andrews S (2010) Fast QC: A quality control tool for high throughput sequence data. Available: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.
[11] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 2010;7(5):335-36.
[12] Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. PNAS. 2011;108(S1):4592-98.
[13] Bik EM, Long CD, Armitage GC, Loomer P, Emerson J, Mongodint EF, et al. Bacterial diversity in the oral cavity of 10 healthy individuals. ISME J. 2010;4:962-74.
[14] Menon T. A metagenomics study of the human oral microbiome in healthy individuals. Paper presented at Microcon 2018, Bangalore India, Abstract 274.
[15] Menon T, Gopalakrishnan SN, Balasubramanian R, Justin SR. Characterisation of the human oral microbiome in patients with coronary artery disease using next-generation sequencing of 16SrRNA amplicons. Indian J Med Microbiol. 2017;35(1):101-04. doi:10.4103/ijmm.IJMM_16_370.
[16] Browne RH. On the use of a pilot sample for sample size determination. Stat Med. 1995,14(17):1933-34. doi.org/10.1002/sim.4780141709

PARTICULARS OF CONTRIBUTORS:
1. UGC BSR Faculty, Department of Microbiology, Dr ALMPGIBMS, University of Madras, Chennai, Tamil Nadu, India.
2. Consultant Pulmonologist, Department of Respiratory Medicine, Madras Medical Mission, Chennai, Tamil Nadu, India.
3. Research Fellow, Department of Microbiology, Dr ALMPGIBMS, University of Madras, Chennai, Tamil Nadu, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Thangam Menon,
Department of Microbiology, DR ALMPGIBMS, Taramani, University of Madras,
Chennai, Tamil Nadu, India.
E-mail: thangam56@gmail.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jun 01, 2020
- Manual Googling: Jul 16, 2020
- iThenticate Software: Aug 29, 2020 (18%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: As declared above
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **May 30, 2020**
Date of Peer Review: **Jul 02, 2020**
Date of Acceptance: **Jul 17, 2020**
Date of Publishing: **Sep 01, 2020**