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The Core and Variable Oral Microbiome of a Group Sri Lankan Oral Squamous Cell Carcinoma (OSCC) Male Patients

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THE CORE AND VARIABLE ORAL MICROBIOME OF A GROUP SRI LANKAN ORAL SQUAMOUS CELL CARCINOMA (OSCC) MALE PATIENTS

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Abstract

Open ended molecular techniques garnered recognition as the mostly applied in microbiome studies. Present study was conducted to identify core and variable microbiome of a group of Sri Lankan OSCC male patients. The universal prokaryotic 16S rRNA gene, with V1- V9 hyper variable regions and the internal transcribed spacer (ITS) with a non-functional DNA segment that is located between 18S, 5.8S and 28S rRNA genes in eukaryotes/fungi are ideal candidates for sequencing after amplification with the specific degenerate primers, for profiling of microbiome. Representative sub sample comprising 25 OSCC cases and 27 fibro-epithelial polyp (FEP) controls as well as 22 OSCC cases and 24 fibro-epithelial polyp (FEP) controls were selected from a large multi-centre field study conducted in Sri Lanka for profiling of bacteriome and mycobiome respectively. DNA were extracted from all frozen tissues according to the manufacturer's instructions. In the present study, the V1 to V3 region of the prokaryotic 16S rRNA gene and fungal ribosomal internal transcribed spacer 2 (ITS2) were used for amplification with degenerate specific primers, followed by sequencing using (Illumina) 2x300 bp chemistry. Raw reads were subjected for pre and final processing procedures to obtain high-quality non-chimeric merged reads. As a result, 89.0 % of bacterial and 97.4 % of fungal processed reads were classified into species. Bacterial classification up to species level was done by prioritized BLASTN-based algorithm and fungal species level using a BLASTN-algorithm with UNITE's named species sequences as reference. Downstream compositional analyses were performed with QIIME and LEFSe software. The core microbiome was consisted of genera of *Streptococcus*, *Rothia*, *Leptotrichia*, *Gemella*, *Capnocytophaga*, *Fusobacterium*, *Prevotella*, *Haemophillus*, *Granulicatella* and *Neisseria* as well as *Candida*, *Malassezia*, *Cladosporium* and *Aspergillus* which were common to FEP controls as well as OSCC cases. Obviously, certain species were differently abundant in the variable microbiome of OSCC patients.

Keywords: Core Oral Microbiome, Variable Oral Microbiome, Universal prokaryotic 16S rRNA gene, fungal ribosomal internal transcribed spacer 2 (ITS2), (Illumina) 2x300 bp chemistry

1. Introduction

The next generation sequencing (NGS) technology has been advented in early two thousand with much higher throughput, than the first generation- Sanger method [1]. Hence, massively parallel high–throughput molecular technologies for instance, have paved the way of viewing microbial ecology by profiling microbial communities in any environment [1,2], at much higher resolution of unprecedented depth and breadth [1,2]. Changes in variable microbiome from health to disease status is utmost importance to understand 'the disease associated microbiome' [3] especially in inflammatory diseases [2], where they play an important role in disease progression via their virulent, immunogenic, carcinogenic and toxic attributes [4,5].

The 16S rRNA, after transcription (r DNA) accomplishes the criteria of an ideal marker gene consisted of highly conserved ubiquitous sequences present in prokaryotes [6, 7] and hyper variable regions (V1-V9) that differ between different taxa by greater or lesser frequency over evolutionary time. As per previous findings, V1-V3 region appears as better in greater phylotype richness and evenness than V3-V4 thus, provides more representative assessment of population diversity and community composition of oral bacteriome [4, 5]. The 18S rRNA has become the house keeping gene of choice at present due to the diversity of ITS, which provides the most effective locus for greater species level phylogenetic placement of fungi and at present commonly used as the fungal 'species barcode' region [8,9]. It has been hypothesized that the core microbiome is stable but the variable microbiome subjected to variations in disease conditions due to genotype, life style related risk habits and physiological differences status pertaining to disease conditions [3, 9].

The oral cavity made up of soft tissues, hard tissues and saliva provides ideal niches for each community which made the diverse oral meta community, seconded only to the gut microbiome [2]. Presence of contaminants deemed another inherent limitation in any metagenomic study [10], which cannot be afforded though and this seems to be the main reason for inconsistencies in the literature of community composition of human microbiome [2,5]. Knowledge gaps in proper surface contamination and clinical relevance in data interpretation need further investigation in compositional profiling of oral microbiome. The objectives of present study was to find out the core and variable microbiome of a group of Sri

Lankan OSCC male patients compared with benign fibro epithelial polyp (FEP) controls

2. Materials and Methods

2.1 Ethical Approval

Ethical approval was obtained from the Faculty Research Committee, Faculty of Dental Sciences, University of Peradeniya, Sri Lanka (FRC/ FDS/UOP/E/2014/32) and Griffith University Human Research Ethics Committee, Australia (DOH/18/14/ HREC).

2.2. Study design, setting, and subjects:

Representative cohort of 25 OSCC cases and 27 fibro epithelial polyp (FEP) controls plus 22 OSCC cases and 24 fibro-epithelial polyp (FEP) controls were selected from a large case control, multi-centre field study conducted in Sri Lanka representing nine ONF units from six Provinces for profiling of bacteriome and mycobiome respectively Cases comprised Sinhala, ≥40-year-old males with histologically confirmed OSCC affecting the buccal mucosa or tongue. The control group consisted of 27 Sinhala males with a clinical diagnosis of FEP also involving the buccal mucosa or tongue. Written informed consent was obtained from each participant.

2.3 Tissue sampling and DNA extraction

Deep tissue pieces (~3 mm3) from incisional biopsies from OSCC cases and excisional biopsies from FEP controls. Tissue samples (~100 mg each) were finely chopped using a sterile blade. Subsequently, DNA extraction from frozen samples (=800C) was then performed using Gentra Puregene Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions (solid tissue protocol) additionally with of 50 units of mutanolysin to optimize the digestion of from Gram positive cell walls.

2.4 *Amplicon library preparation and sequencing:*

With the degenerate prokaryotic primers 27 FYM (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GW ATTACCGCGGCKGCTG3'), the V1-V3 region of the 16S rRNA gene was amplified. Library preparation, indexing, and sequencing were performed at the Australian Centre for Ecogenomics (University of Queensland, Australia) with thev3 2 × 300–bp chemistry on a MiSeq platform (Illumina). Library preparation, indexing and sequencing were performed at the Australian Centre for Ecogenomics (University of Queensland, Australia) with the v3 2 × 300–bp chemistry on a MiSeq platform (Illumina). The fungal primers ITS3-F (5'-GCATCGATGAAGAACGCAGC-3') and ITS4-R (5'TCCTCCGCTTATTRATATGC-3') were used to target ITS2, while primers β-actin-g DNA-F (5'TCCGCAAAGACCTGTACGC-3') andβ-acting DNA-R (5'CAGTGAGGACCCTGGATGTG-3') were used to amplify the β-actin gene.

2.5 Data processing:

Preprocessing of data: primer trimming, merging, quality filtration, alignment, and chimera check were performed as detailed previously for prokaryotic and fungal raw sequencing reads [4, 5, 7]. The high-quality non chimeric merged reads were classified upto species level using BLASTN searched against 4 databases of 16S rRNA prokaryotic gene reference sequences at alignment coverage and percentage identity ≥98% and then assigned species-level taxonomy of the hit with highest percentage identity and bit score, and belonging to the highest-priority reference set. Reads with no matches at the set cutoffs were subject to de novo operational taxonomic unit calling and assigned to the closest species [4, 5, 7]. Fungal species level identification was done using modified BLASTN-based algorithm and UNITE's database v7.1 (https://unite.ut.ee/repository.php; 22) [4,5,7]. *2. 5*

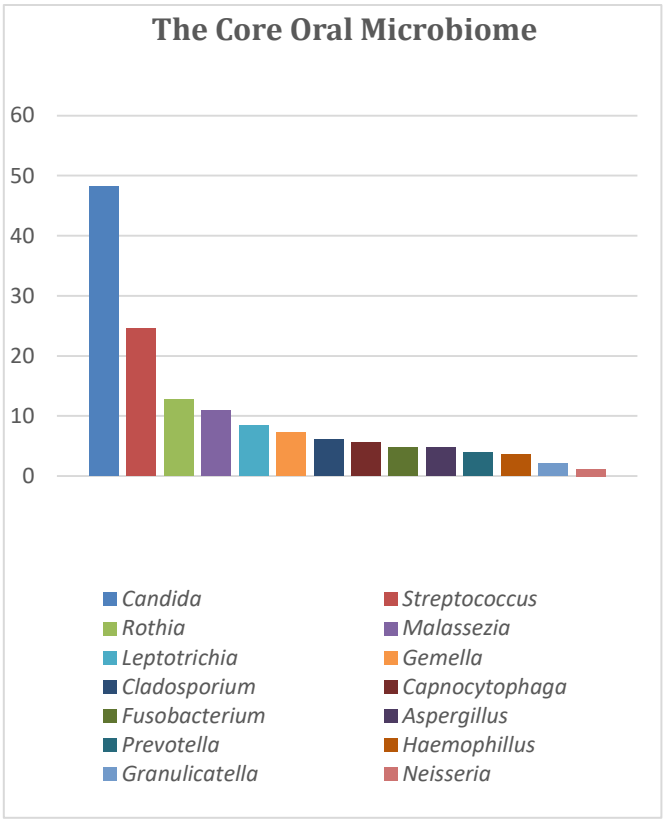
2.6 Compositional and Statistical Analysis: Compositional analysis of bacteriome and mycobiome was performed using QIIME (Quantitative Insights into Microbial Ecology) [11] as described previously. Statistical analysis of differentially abundant taxa, genes, and pathways between the cases and controls were sought with linear discriminant analysis effect size (LEFSe) and G test [5].

3. Results and Discussion

Analyzed sequencing data were presented here to view the differences in 'core' and 'variable' oral microbiome of a group of Sri Lankan male subjects in snap shot.

3.1 The core oral microbiome of a group of Sri Lankan male subjects

Figure 1; The main genera of core oral microbiome common to FEP and OSC



The core microbiome, consisted of *Candida, Streptococcus, Rothia, Malassezia,* and *Leptotrichia* with relative abundances of approximately 50%, 25%, 13% , 11% and 8% respectively.

3.2 The variable oral microbiome of a group of Sri Lankan OSCC male patient

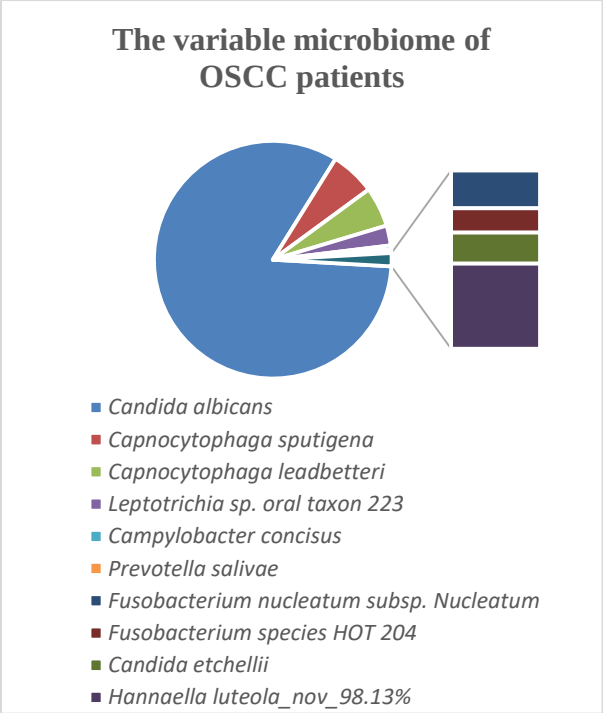


Figure2: The main species of variable oral microbiome of OSCC patients

The patho biont species namely *C. albicans*, *C. sputigena*, *C. leadbetteri* and *Leptotrichia, sp.oral taxon 223* were the main species of variable microbiome of Sri Lankan, OSCC male patients. They were able to thrive well in oral cancer tissues due to dysbiotic condition occurred breaking the homeostasis.

4. Conclusion: It seemed the core microbiome was common to both benign FEP and malignant OSCC Sri Lanka subjects. However, a clear variation in species level was observed by the variable microbiome of Sri Lankan OSCC patients.

Recommendations: This was the first and foremost study conducted on oral microbiome of Sri Lankan subjects. Hence, further research work based on this important finding is strongly recommended.

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References

1.. Siqueira JF Jr., Fouad AF, Rocas IN. Pyrosequencing as a tool for better understanding of human microbiomes. J Oral Microbiol 2012; 4, 10743, doi: http://dx.doi.org/10.3402/jom. v4i0.10743

2. Perera M, Al-hebshi NN, Speicher DJ, Perera I, Johnson NW. 2016. Emerging role of bacteria in oral carcinogenesis: a review with special reference to perio-pathogenic bacteria. J Oral Microbiol. 8:32762

3. Zarco MF, Vess TJ, Ginsburg GS. (2012). "The oral microbiome in health and disease and the potential impact on personalized dental medicine." Oral diseases 18(2): 109-120.

4. Perera M, Al-hebshi NN, Perera. I, Ipe D, Ulett GC, Speicher DJ, Chen T, Johnson, NW (2018) "Inflammatory bacteriome and oral squamous cell carcinoma." Journal of dental research. 97(6): 725-732

5. Al-hebshi NN, Nasher AT, Maryoud MY, Homeida HE, Chen T, Idris AM, Johnson (NW2017). "Inflammatory bacteriome featuring *Fusobacterium nucleatum* and *Pseudomonas aeruginosa* identified in association with oral squamous cell carcinoma." Scientific reports 7 (1): 1-10.

6. Dollive, S., Peterfreund GL, Sherrill-Mix, S. Bittinger, K and Sinha, R et al., (2012). "A tool kit for quantifying eukaryotic rRNA gene sequences from human microbiome samples." Genome biology 13(7): 1-13.

7. Perera M, Al-hebshi NN, Perera I, Ipe D, Ulett GC, Speicher DJ, Chen T, Johnson NW. (2017). A dysbiotic mycobiome dominated by Candida albicans is identified within oral squamous-cell carcinomas. J Oral Microbiol. 9(1):1385369

8. Dupuy AK, David MS, Li L, et al (2014). "Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of Malassezia as a prominent commensal." PLoS One 9(3): e90899.

9. Glassing, A., et al. (2016). "Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples." Gut pathogens 8(1): 1-12.

10. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 7(5):335–336