Low abundance of Capnophiles in the saliva of Oral Squamous Cell Carcinoma patients- a metagenomic analysis

Thematic area: Microbiology

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Abstract

Objective: Alterations in microbiome have been linked to Oral squamous cell carcinoma (OSCC) by few earlier studies. We aimed to assess such changes in OSCC patients. **Methods**: *16S rRNA* metagenomic analysis of saliva samples from OSCC patients and health were performed by the latest next generation sequencing (MinION nanopore platform) using pan-eubacterial primers that amplify the entire *16S rRNA* gene. The sequences were analysed with MG-RAST. **Results**: Capnophilic Capnocytophaga species, (*Capnocytophaga gingivalis Capnocytophaga ochracea, Capnocytophaga sputigena, Capnocytophaga cynodegmi* and *Capnocytophaga canimorsus*) were found to be in low abundance among OSCC patients compared to the healthy subjects. **Conclusion:** These findings suggest the association of Capnocytophaga species to health.

Key words:

Capnocytophaga, oral microbiota, oral squamous cell carcinoma, next generation sequencing*, 16S rRNA* metagenomic analysis,

<u>**Introduction**</u>

Oral squamous cell carcinoma (OSCC), a subgroup of head and neck squamous cell carcinoma frequency accounts for more than 90% of all oral cancer. Globally OSCC is the foremost cause for mortality. Cases of OSCC are on the raise in India and Indian subcontinent countries. The etiology of OSCC is multifaceted and comprises of many factors. Tobacco habit is one of the major risk factor for OSCC. [1] Additional probable risk factors may comprise oral microbiota. [2,3] Human saliva is composed of complex microbiota. [4,5]

Pathogenic microbiota in particular the bacterial species is very much linked with inflammation and cancer progression. [6,7] Anaerobes were earlier reported to be dominating aerobes on the lesions of OSCC by culture methods. Smruti *et al*., 2012 [8] has reported an increase of *Capnocytophaga gingivalis, Prevotella melaninogenica,* and *Streptococcus mitis* in the saliva of OSCC patients. The present study through metagenomic analysis highlights the low abundance of capnophilic bacteria in the saliva of Oral squamous cell carcinoma patients.

Materials and Method

This study was approved by the Institutional Ethics Committee of Sree Balaji Dental College & Hospital, Chennai (Ethics approval No: SBDCH/IEC/ 09/2016/15). Written informed consent was obtained from the patients and healthy individuals after explaining the study proposal. The study population included patients with OSCC (n=5) and healthy subjects (n=5). Unstimulated whole saliva was collected by the "draining" method. The subject's head was tilted forward so that saliva moved toward the anterior region of the mouth and the pooled saliva (2 ml) was collected into a wide-mouthed sterile container. [9] [Oberg et al] The sample was immediately stored at -80ºC.

DNA Extraction

DNA extraction was achieved with a XPRESS DNA Saliva kit (MagGenome/ Cat no: MG18Sa 25). The DNA was extracted from the saliva. Quality and quantity of extracted DNA were assessed using Qubit 4.0 Fluorometer with standards.

PCR and *16S rRNA* Gene Sequencing

A PCR targeting the complete *16S rRNA* gene was performed for the samples with 25µL reaction volume consisting of broad-range *16S rRNA* primers as described by Weisburg et al., (1991). The PCR mixture contained (10 pM of each primer), 10× PCR buffer, 10 mM of dNTP mix, 1 units Taq DNA polymerase, DNA template (0.1-1 µg of DNA) and PCR grade water. Initial denaturation at 95ºC for 3 min, followed by 37 cycles of denaturation at 95ºC for 0.30 min, primer annealing at 55ºC for 0.30 min, extension at 72ºC for 1 min, final extension at 72ºC for 7 min were the cycling condition. *16S rRNA* metagenomic analysis of saliva samples were performed by the latest next generation sequencing (Oxoford nanopore technology (3rd generation) MinION nanopore platform).

Sequences in the form of FASTQ files were uploaded to MG-RAST server running version 4.0.3. Bacterial species abundances were unveiled and exported from MG-RAST using RDP database with Best Hit Classification.

Results:

Capnocytophaga species (*Capnocytophaga gingivalis Capnocytophaga ochracea, Capnocytophaga sputigena*) were found to be abundant among the healthy subjects compared to OSCC group. *Capnocytophaga cynodegmi* and *Capnocytophaga canimorsus* species was found to occur in healthy subjects (Fig 1). Low abundance of *C. cynodegmi* and *C. canimorsus was* observed among OSCC patients. The bacterial abundance of the five different *Capnocytophaga* species is shown in Table 1. In two OSCC patients saliva samples complete absence of *C. cynodegmi* was noted. Total absence of *C. canimorsus* was observed in one saliva sample of OSCC patient. In the family Flavobacteriaceae, the dominant genera was *Capnocytophaga* in both OSCC and healthy subjects. (Fig 2)

Discussion:

The major risk factors for OSCC is multifactorial viz., tobacco usage in the form of smoking or chewing, areca nut/betel quid chewing, alcohol intake and certain viruses like HPV. Although scientific advancement in therapy has improved the survival rate of OSCC patients, nevertheless more than 50% of patients show poor prognosis. Currently researchers are showing interest to study the role of bacteria in OSCC. This interest may be attributed to diverse niche of the oral cavity and complex nature of the oral microbiome. *16S rRNA* metagenomic analysis with next generation sequencing has helped in exploring the microbiome changes in a diseased condition. The present study reports a shift in *Capnocytophaga* species in OSCC.

The genus *Capnocytophaga* is a Gram-negative bacillus. They are encountered as normal commensal in the oral cavity of mammals. They are slow growing, facultative anaerobes which grows luxuriantly in the presence of 5-10% of CO_2 and hence referred capnophilic. [10] The result of the present study is a significant finding with regard to bacterial abundance in the salivary samples of OSCC and healthy subjects. The capnophilic bacterial species belonging to the phylum Bacteroidetes was found in less abundance in the saliva of OSCC patients compared to the saliva of healthy subjects. A significant reduction in these capnophilic groups (*C. gingivalis, C. ochracea, C. sputigena, C. cynodegmi* and *C. canimorsus*) of bacterial species suggests their role in health.

The low abundance of *C. gingivalis* in the saliva of OSCC patients compared to healthy subjects observed in the present study is not in concurrence with an earlier study, where the author reports an increase of *C. gingivalis in the* OSCC patients. [8] *Capnocytophaga* abundance was not reported in an earlier study among OSCC patients. [11] The genus richness in the family Flavobacteriaceae was largely contributed by *Capnocytophaga* in both OSCC and healthy subjects, which was not observed in the previous studies. In spite of such genus richness, *Capnocytophaga* was found in more abundance in health compared to OSCC patients. (Fig 2) The family Flavobacteriaceae showed low genus diversity among healthy subjects compared to OSCC patients. (Fig 2)

Conclusion:

Studying the shifts in the oral microbiome may help in developing a diagnostic kit to identify the biomarkers the help in predicting OSCC. The present study has revealed a significant reduction in the Capnocytophaga species (*C. gingivalis, C. ochracea, C. sputigena, C. cynodegmi* and *C. canimorsus*) in the saliva of OSCC patients compared to healthy subjects suggesting their role in health. *C. cynodegmi* and *C. canimorsus* absence or

very low abundance in OSCC signifies them as a promising probiotic in maintaining a healthy oral cavity. The results may be further strengthened by exploring the functional pathways of the capnophilic bacterial species.

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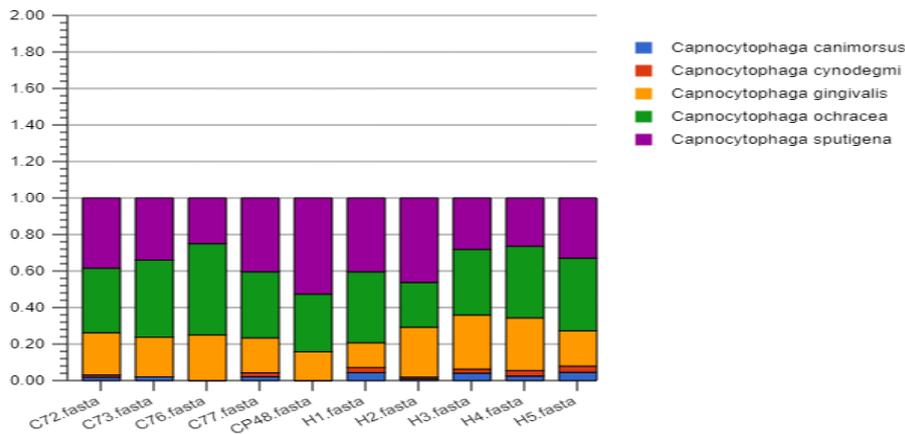Fig-1: Stacked bar chart showing the *Capnocytophaga* species abundance

Fig-2: Stacked bar chart showing the *Capnocytophaga* genus richness in the family Flavobacteriaceae
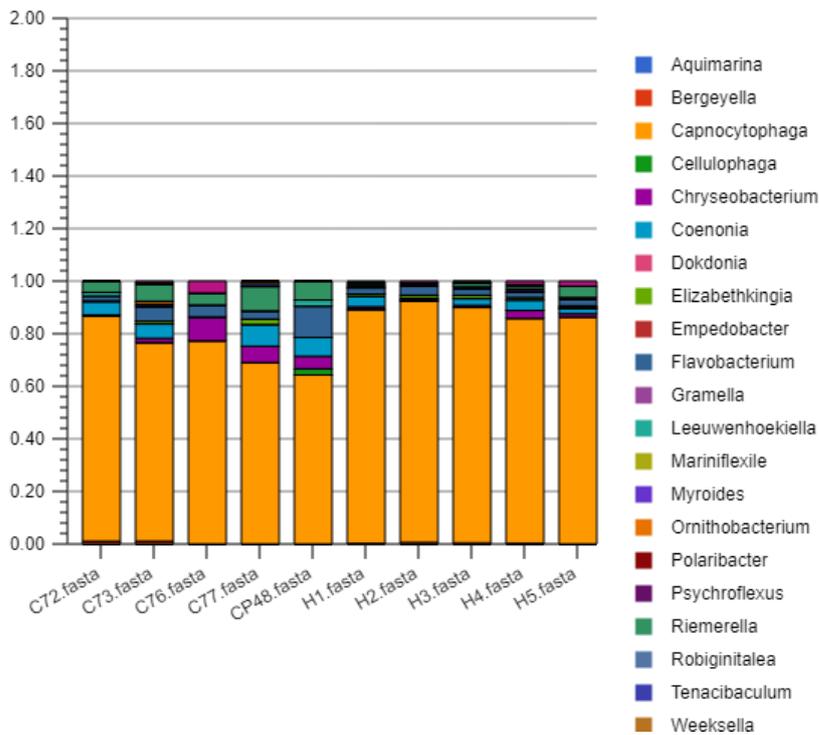

Table-1: *Capnocytophaga* species count

Capnocytophaga species	C72	C73	C76	C77	C48	H1	H2	H3	H4	H5
C. canimorsus	15	16	0	8	3	**155**	**8**	**28**	**11**	**14**
C. cynodegmi	4	1	0	1	0	**55**	**1**	**8**	**6**	**3**
C. gingivalis	75	39	6	12	7	**374**	**57**	**154**	**105**	**39**
C. ochracea	63	43	8	20	6	**687**	**30**	**123**	**82**	**46**
C. sputigena	84	40	3	26	11	**703**	**59**	**106**	**56**	**37**

C72,73,76,77,48- OSCC patients, H1,2,3,4,5 - Healthy subjects