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ORIGINAL ARTICLE

The oral microbiome community variations associated with normal, potentially malignant disorders and malignant lesions of the oral cavity

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Abstract

The human oral microbiome has been known to show strong association with various oral diseases including oral cancer. This study attempts to characterize the community variations between normal, oral potentially malignant disorders (OPMD) and cancer associated microbiota using 16S rDNA sequencing. Swab samples were collected from three groups (normal, OPMD and oral cancer) with nine subjects from each group. Bacteria genomic DNA was isolated in which full length 16S rDNA were amplified and used for cloned library sequencing. 16S rDNA sequences were processed and analysed with MOTHUR. A core oral microbiome was identified consisting of Firmicutes, Proteobacteria, Fusobacteria, Bacteroidetes and Actinobacteria at the phylum level while *Streptococcus*, *Veillonella*, *Gemella*, *Granulicatella*, *Neisseria*, *Haemophilus*, *Selenomonas*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, *Porphyromonas* and *Lachnoanaerobaculum* were detected at the genus level. Firmicutes and *Streptococcus* were the predominant phylum and genus respectively. Potential oral microbiome memberships unique to normal, OPMD and oral cancer oral cavities were also identified. Analysis of Molecular Variance (AMOVA) showed a significant difference between the normal and the cancer associated oral microbiota but not between the OPMD and the other two groups. However, 2D NMDS showed an overlapping of the OPMD associated oral microbiome between the normal and cancer groups. These findings indicated that oral microbes could be potential biomarkers to distinguish between normal, OPMD and cancer subjects.

Keywords: oral bacteria, oral cancer, Malaysia, 16S cloned library, DGGE

INTRODUCTION

Oral cancer is a subset of head and neck cancer which is a type of cancer that occurs in the oral cavity affecting the lips, tongue, gingiva, floor of mouth, palate and other related structures (International Classification of Diseases 10th edition codes C00-06).[1,2]

Globally, it was estimated to be the 15th most common cancer with higher incidences among males in 2012.[3] The estimated incidence and death from oral cancer saw a global increment of 14.2% and 13.9% respectively from 2008 to 2012.[3,4] Two-thirds of the cases occurred in developing countries and it is a major persistent health issue in parts of South Central Asia and South East Asia, where it was estimated to be one of five most common cancer between 2008 and 2012.[3-5] In parts of these regions, oral cancer was shown to comprise up to 40% of all cancer cases.[6] In high risks countries such as Papua New Guinea, Pakistan, Maldives, Sri Lanka and India, it was one of the three most common cancers.[3]

In Malaysia, oral cancer is the third most common head and neck cancer, affecting the elderly population and with equal risk for both genders.[7] It is most common among Indians

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regardless of different subsites such as mouth and tongue.[8,9]

Oral cancer is a persistent health issue due to its asymptomatic early stages and the absence of reliable diagnostic techniques.[10] These factors have been contributed to the late detection of advanced cancer stages thereby resulting in reduced effectiveness of treatment and the post-surgery life quality as well as poor survival rate. [5,11-13] Early detection and treatment could cure the cancer, improve the survival rate, and improve the quality of life due to less debilitating treatments.[14,15]

Tobacco, alcohol and betel quid are known oral cancer risk factors; emerging aetiological factors include bacterial, viral or fungal infections.[16-18] About 20% of the human tumours are preceded by infections or inflammations while many oral cancers were found to develop from oral potentially malignant disorders (OPMD) such as leukoplakia, erythroplakia and erythroleukoplakia.[19-21] These lesions are associated with chronic inflammation, which has been observed at various stages of oral cancer and epithelial dysplasia, which is known to be associated with risk of malignancy.[22]

In addition to cancer associated inflammation, certain microbial species have been found to be cancer associated. For instance, *Prevotella melaninogenica*, *Capnocytophaga gingivalis* and *Streptococcus mitis* appear to be significantly elevated in tumour tissues compared to normal counterparts from the same patient.[23] Besides that, the salivary microbiome of oral cancer patients formed a distinct cluster separated from the normal group in a study using the Denaturing Gradient Gel Electrophoresis (DGGE) fingerprint technique.[24] It has been suggested that this association between oral cancer and microbiome can be exploited for the development of diagnostic tools since bacteria is able to selectively bind to and colonize mucosal surfaces similar to a "lock and key" mechanism.[23,25]

Some oral tumours are derived from oral potentially malignant disorders (OPMD), which is a group of disorders described as genetically mutated oral epithelial cells with or without clinical and histomorphological abnormal appearances that are malignant transformable.[26] OPMD with subtle abnormalities present diagnostic challenges while the potential for multifocal cancer development not only complicates prognosis but also reduces treatment effectiveness.[27-29] The associated oral microbiome was not addressed in previous studies which otherwise could uncover OPMD associated oral microbes that have the potential to be biomarkers for the detection of genetically abnormal oral mucosa prior to the early oral cancer stage.[15,23-26]

Therefore, this study aims to identify oral cancer associated microbiota variations through the cross sectional comparison of the normal, OPMD and cancer 16S cloned libraries with the intention to identify OPMD and cancer associated microbial profiles with potential medical value. The results suggest several oral microbial species are associated with certain oral clinical states.

MATERIALS AND METHODS

Subject recruitment

Ethical approvals for the recruitment of normal volunteers and oral cancer patients were granted by University Tunku Abdul Rahman (UTAR) Scientific & Ethical Review Committee (U/SERC/03/2011) and Faculty of Dentistry Medical Ethics Committee of the University of Malaya (UM) respectively (DF OP1208/0056(L)). Normal (n=9) subjects were recruited, with written informed consent, from UTAR while OPMD (n=9) and cancer (n=9) samples were collected from the biobank of Oral Medicine/Oral Surgery Clinic at the Faculty of Dentistry (coordinated by the Oral Cancer Research and Coordinating Center (OCRCC), UM). Normal subjects and patient samples were selected based on the criteria in Table 1. The size of all subject groups was standardized according to the number of matched OPMD samples that can be collected from the biobank.

Two swabs (Millipore) were collected from the normal subject by rubbing against oral regions that cover the entire oral cavity (Table 2). Swabs were dipped into 400 µL of preservation buffer (50 mM Tris, pH 8.0, 50 mM EDTA, 50 mM sucrose, 100 mM NaCl, 1% SDS) and stored at ambient temperature prior to DNA extraction.[30]

DNA extraction

DNA extraction from swabs was done with a commercial kit (EURx) with some modifications to the manufacturer's protocol to compensate for the addition of the preservation buffer as the starting material. Briefly, the volume of proteinase K, Sol S buffer and absolute ethanol were doubled while the incubation period of Proteinase K digestion was prolonged to overnight with shaking prior to steps involving the spin column.

TABLE 1: Selection criteria

Inclusion criteria	Groups	Exclusion criteria
• Nationality is Malaysia • Minimum age of 20 years old • Minimum teeth number of 20	All	• Currently having malaise, pregnancy or lactation • Infected with HIV • Under antibiotic medication within the past 3 months • Involve surgery, chemotherapy or radiation treatment within the past 1 month
	Normal	• Currently with gingival health issues such as ulcers and inflammations
• Diagnosed with OPMD or cancerous lesions	OPMD and Oral Cancer	• Feel discomfort during sampling procedures and withdraw from volunteering

DNA yield and purity were determined spectrophotometrically with a NanoPhotometer (IMPLEN). The extracted DNA was stored at -80°C prior to downstream molecular processes.

16S PCR

The D88 (GAG AGT TTG ATY MTG GCT CAG) and E94 (GAA GGA GGT GWT CCA RCC GCA) primer pair was selected for use in this study to produce near full length 16S PCR amplicons based on their extensive usage in oral microbiome related studies.[25,31-33] The PCR mixture contained around 35 ng DNA template, 1X $MgCl_2$ free buffer, 0.5 mM $MgCl_2$, 0.5 µM of each forward and reverse primer, 0.2 mM of dNTPs mixture and 1.5 U Taq polymerase (Intron Biotechnology) in 50 µL. All PCRs were carried out in the Veriti® 96-Well Thermal Cycler (Applied Biosystems) with 8 minutes of denaturation at 95ºC, followed by 25 cycles of denaturation at 95ºC (45 seconds), annealing at 63ºC (60 seconds) and extension at 72ºC (90 seconds), and a final 72ºC extension step for 10 minutes. 16S PCR amplicons were examined with 1% agarose gel and purification using the MEGAquick-spin (iNtRON).

16S cloned library

Purified 16S PCR amplicons were cloned into pSTBlue-1 AccepTor Vector (Novagen) which was introduced into NovaBlue Singles™ Competent Cells according to the manufacturer's protocol. Cloned libraries were screened for the correct insert size of approximately 1.5kb with colony PCR prior to further analysis.

RFLP analysis and DNA sequencing

Cloned libraries were screened for distinct RFLP patterns with *Msp*I and *Hha*I digestion for 1 hour at 37ºC (Thermo Scientific). Colonies with similar RFLP patterns were grouped together and the amount of colonies per RFLP group was used as abundance data.[34]

Transformed bacteria were cultured overnight and plasmid was extracted using High-Speed Plasmid Mini Kit (Geneaid) according to the manufacturer's protocol. All purified plasmid were sent to Bioneer Inc. for partial 16S rDNA sequencing that targets variable regions from V6 to V9.

The partial 16S sequences obtained in this study have been deposited in the GenBank database under the accession number KP294530 to KP294905.

Bacteria sequence identification and cloned library analysis

16S primer sequences were trimmed with Sequence Scanner 1.0 (Applied Biosystem) and were searched against the Human Microbiome Project Data Analysis and Coordination Center (HMP-DACC) which is available at http://www.

TABLE 2: Selected oral regions for swab sampling

Swabs	Oral regions
1	Tongue, mouth floor and lower gingival
2	Buccal, soft and hard palate as well as upper gingival

TABLE 3: Formulae

Calculations	Formula	Symbol descriptions
Good's coverage	(1-(n/N))100	n is the singletons amount while N is the total amount of OTUs
Shannon Index	$-\Sigma p_i \ln p_i$	p_i is the ratio of specific OTUs
Effective species number of Shannon index	$e^{D_{Shannon}}$	$D_{Shannon}$ is the Shannon index
Simpson Index	Σp_i^2	p_i is the ratio of specific OTUs
Effective species number of Simpson index	$1/D_{Simpson}$	$D_{Simpson}$ is the Shannon index

hmpdacc.org/resources/blast.php. Representative sequences were assigned to bacteria species with the highest bit score and lowest E-values.

All representative sequences were processed using the MOTHUR software with some modifications.[35]

Diversity estimation

Rarefaction curve and library coverage were calculated using the "rarefaction.single" and "collect.single" commands respectively together with Shannon and inverse Simpson indexes with the MOTHUR software. Shannon index were converted to effective species number (Table 3) while the value of inverse Simpson index remained unchanged since it is the effective species number of itself.[36]

Community comparisons

Common Operational Taxonomic Units (OTU) that can be found across all subject groups were identified. Besides, shared OTUs that are overlapped across any two subject groups as well as unique OTUs that can be found in one subject group were also tabulated.

Individual cloned libraries were organized based on RFLP abundance records and normalized prior to NMDS ordination and AMOVA. NMDS ordination was used to illustrate the similarities between different community structures which was calculated using MOTHUR software and plotted in R language environment using rgl package.[37,38] The significant difference of genetic diversity between pooled and within two communities was tested with AMOVA using the MOTHUR software.[39]

Phylogenetic analysis

Representative sequences were aligned by ClustalW with some 16S reference sequences from NCBI Nucleotide database. Phylogenetic tree was generated by Neighbour Joining method using Mega 6.0 software.[40]

RESULTS

Demographic data of subjects

The subject groups consisted of Malay, Chinese and Indian ethnicities. The male to female ratio of the normal and cancer group was almost equal while there were more female OPMD patients (Table 4). The average age of the cancer group was the highest, followed by the OPMD group and finally the normal group (Table 4). The smoking and drinking habit of all three subject groups were almost similar (Table 4). The OPMDs were confirmed histologically to be epithelial dysplasia while the oral cancer was confirmed histologically to be squamous cell carcinoma.

TABLE 4: Subject demographic data

Subject groups	Normal	OPMD	Cancer
Gender:			
Male	4	2	4
Female	5	7	5
Average age	40	54	60
Smoking habit	1	1	2
Drinking habit	0	1	1

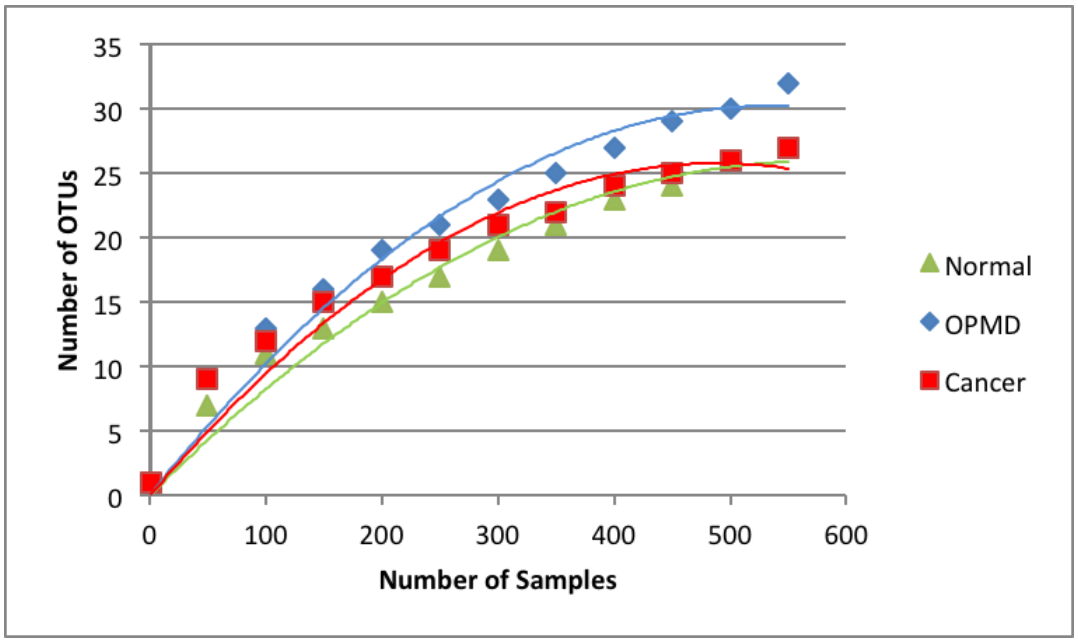


FIG. 1: Rarefaction curve of normal, OPMD and cancer groups. Normal associated group symbolized as green triangles, OPMD associated group symbolized as blue squares and cancer associated communities symbolized as red squares

Diversity metrics

The plateau stage of rarefaction curves (Fig. 1) and Good's coverage of around 97% (Table 5) indicated that the number of colonies screened was sufficient to represent the normal, OPMD and cancer cloned libraries.

The effective species number of both Shannon and Simpson indexes were highest for the cancer group, followed by OPMD and finally the normal group (Table 5). The species richness was the highest in the OPMD group and similar between the normal and cancer group (Table 5).

Bacterial profiles of normal, OPMD and cancer associated oral conditions

A total of five bacteria phyla were detected in all subject groups, with Firmicutes as the predominant phylum followed by Proteobacteria, Fusobacteria, Bacteroidetes and Actinobacteria (Fig. 2). Certain phyla was found to be of higher abundance in particular oral condition (Fig. 2).

Twelve bacterial genera were found across all subject groups with *Streptococcus* being the predominant genus (Fig. 3). *Veillonella*, *Gemella*, *Granulicatella* and *Neisseria* form another portion of this common oral microbiome fraction with a total relative abundance ranging between 27.3% to 32.2% (Fig. 3). The remaining seven bacterial genera of *Haemophilus*, *Selenomonas*, *Fusobacterium*, *Leptotrichia*, *Prevotella*, *Porphyromonas* and *Lachnoanaerobaculum* only constitute a small proportion with a total relative abundance of less than 5% (Fig. 3). The relative abundance of *Streptococcus* and *Veillonella* were higher in the normal group while the relative abundance of *Neisseria*, *Gemella* and *Granulicatella* were higher in the patient groups (Fig. 3).

The total amount of bacterial species identified in this study is about 80 species, comparable with

TABLE 5: Good's coverage, species richness and effective species number of Shannon and Simpson indexes

Groups	Good's coverage, %	Species richness	Effective species number of Shannon Index	Inverse Simpson Index
Normal	97.22	29	3.67	2.15
OPMD	96.96	35	4.96	2.66
Cancer	98.05	28	4.99	2.72

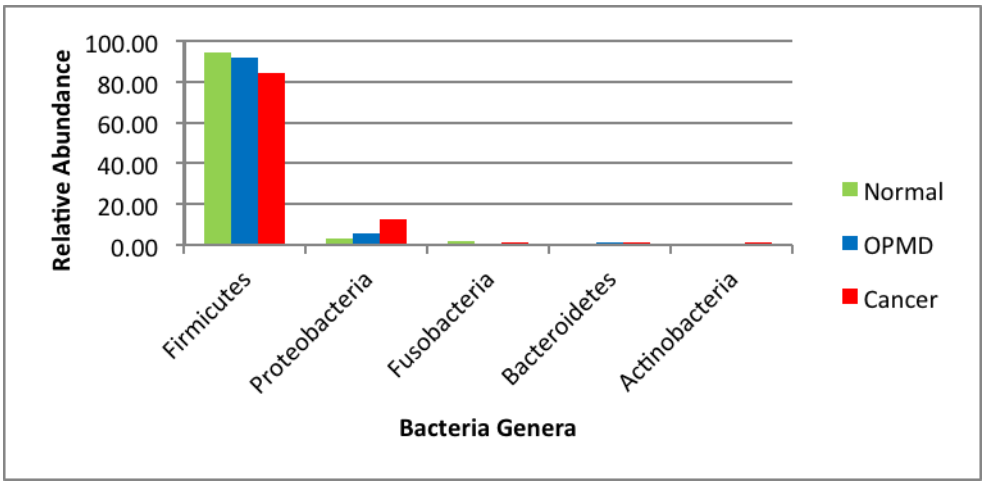


FIG. 2: The relative abundance of 5 major bacteria phyla in normal, OPMD and cancer groups. Normal associated group symbolized as green triangles, OPMD associated group symbolized as blue squares and cancer associated communities symbolized as red squares

previous similar study.[15] Some bacterial species were unique to a single oral conditions and some were found shared between 2 oral conditions. Of these, several bacterial species were found to have consistent association to certain oral conditions previously reported (Table 6).

Association between bacteria phylogenetic groups and oral conditions

The phylogenetic trees (Figs. 4a-4f) revealed that bacteria groups were associated to normal and diseased oral clinical samples. Besides, four unclassified OTUs were identified since they did not cluster with any closest reference sequences. Two were closely related to Ruminococcaceae (Fig. 4a), one was closely related to Clostridiales (Fig. 4b) while one was closely related to Veillonellaceae (Fig. 4a).

Bacteria groups that have association to only normal oral conditions were mainly identified in the Firmicutes phyla and they included the predominant *Streptococcus* and *Veillonella* that had higher relative abundance in the normal oral cavity, and Lachnospiraceae that was detected only in the normal condition (Figs. 4a & 4b).

Bacteria groups that showed an association with only OPMD or cancer or both diseased oral conditions were found across all bacteria

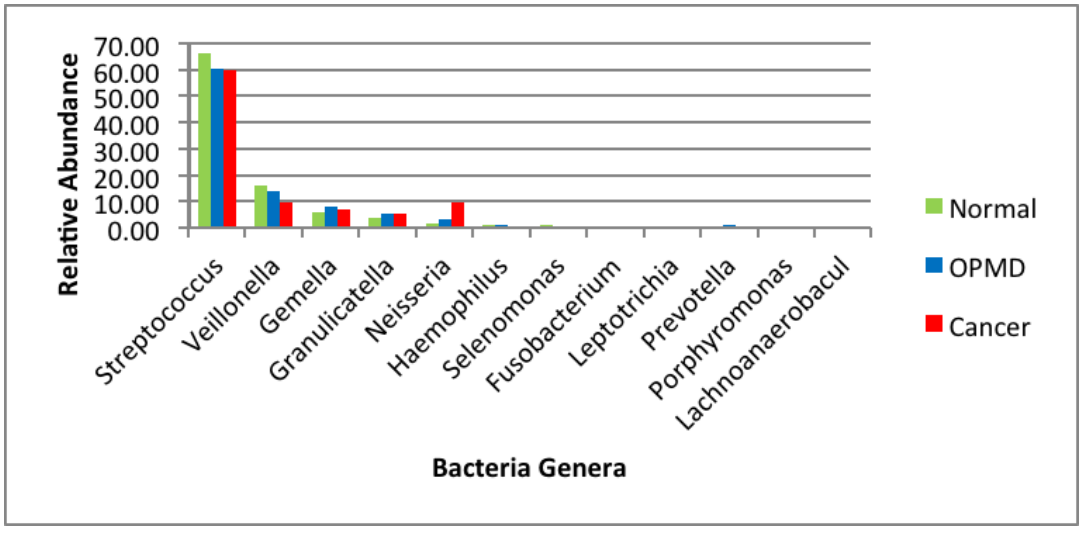
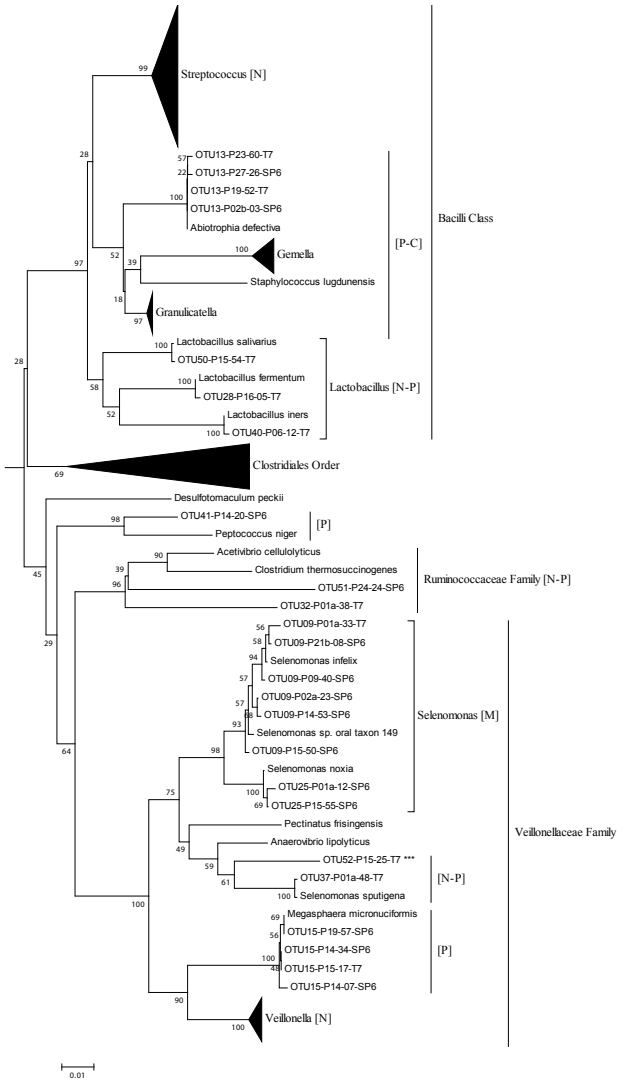


FIG. 3: The relative abundance of common bacteria genera that were found in all subject groups. Normal associated group symbolized as green triangles, OPMD associated group symbolized as blue squares and cancer associated communities symbolized as red squares

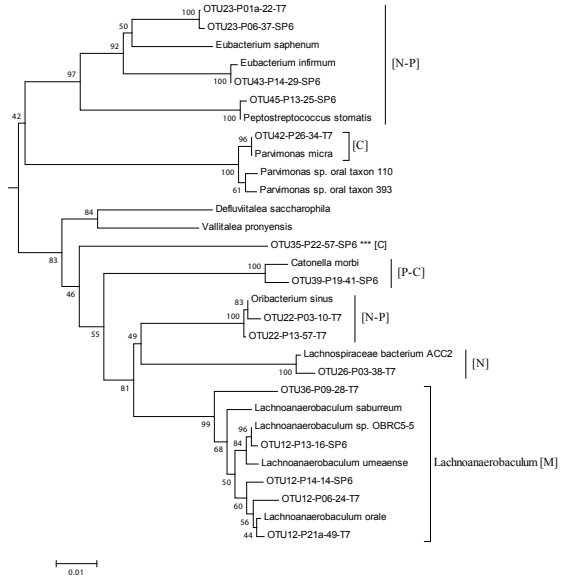
TABLE 6: Bacteria Species or Groups identified to have consistent association to certain oral health conditions in this study and previous published studies

Bacteria Species / Groups identified in the current study	Association to oral health conditions	References
Normal		
*Eubacterium saphenum**	normal saliva	Mager *et al* 2005[23]
*Lactobacillus iners**	NT tissues	Pushalkar *et al* 2012[15]
*Selenomonas sputigena**	NT	Pushalkar *et al* 2012[15]
Actinomyces odontolyticus	cancer saliva	Mager *et al* 2005[23]
Lautropia mirabilis	isolated from mouth of HIV infected patient	Rossman *et al* 1998[60]
Stomatobaculum logum	isolated from oral cavity	GIDEON database[56]
Capnocytophaga leadbetteri	isolated from oral cavity	GIDEON database[56]
Lachnoanaerobaculum saburreum	isolated from dental plaque	GIDEON database[56]
Normal – OPMD		
Prevotella melaninogenica, *Prevotella veroralis*	T; NT; normal saliva	Hooper *et al* 2007[25]; Iwamoto *et al* 2010[61]; Pushalkar *et al* 2012[15]
Oribacterium sinus	N/A	
Selenomonas noxia	N/A	
OPMD		
Megasphaera micronuciformis	T	Pushalkar *et al* 2012[15]
Prevetella nigrescens	cancer saliva	Mager *et al* 2005[23]; Pushalkar *et al* 2011[24]
Eubacterium infirmum	T	Pushalkar *et al* 2012[15]
Lactobacillus fermentum	T	Pushalkar *et al* 2012[15]
Peptostreptococcus stomatis	T	Pushalkar *et al* 2012[15]
Lactobacillus salivarius	oral probiotic	Iwamoto *et al* 2010[61]
Prevotella multiformis	periodontitis	Sakamoto *et al* 2005[62]
Actinomyces urogenitalis	N/A	
Bacteroides clarus	N/A	
Rothia aeria	N/A	
Peptococcus niger	N/A	
OPMD – Cancer		
*Rothia mucilaginosa**, *Rothia dentocariosa**	cancer saliva	Pushalkar *et al* 2011[24]
*Catonella morbi**	T	Pushalkar *et al* 2012[15]
Prevotella pallens	NT tissues	Pushalkar *et al* 2012[15]
Aggregatibacter segnis	isolated from saliva of HIV infected patients	Li *et al* 2014[63]
Haemophilus paraphrohaemolyticus	N/A	
Haemophilus haemolyticus	N/A	
Abiotrophia defective	N/A	
Cancer		
*Camplylobacter showae**	T	Pushalkar *et al* 2012[15]
Capnocytophaga sputigena	N/A	
Parvimonas micra	NT	Pushalkar *et al* 2012[15]
Prevotella salivae	NT	Pushalkar *et al* 2012[15]
Corynebacterium matruchotii	N/A	
Normal – Cancer		
Campylobacter gracilis	oral lichen planus	Ertugrul *et al* 2013[64]

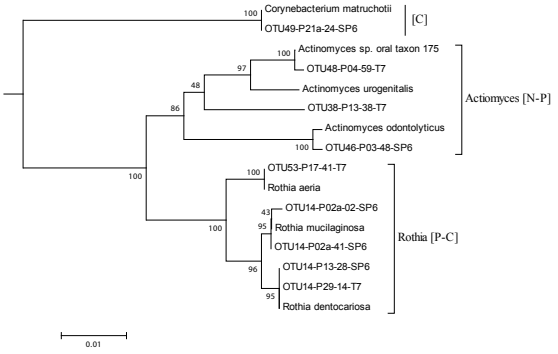
Symbols: * – bacterial species with consistent affiliation with particular oral condition; T – tumour tissues; NT – non-tumour tissues; N/A – information not available



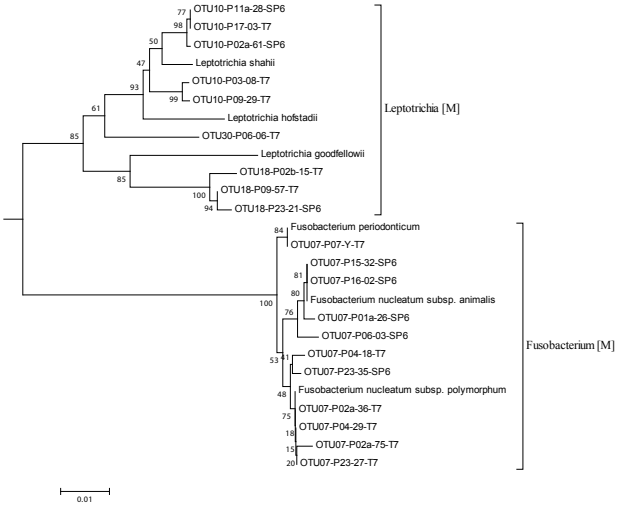
a. Firmicutes Phylum



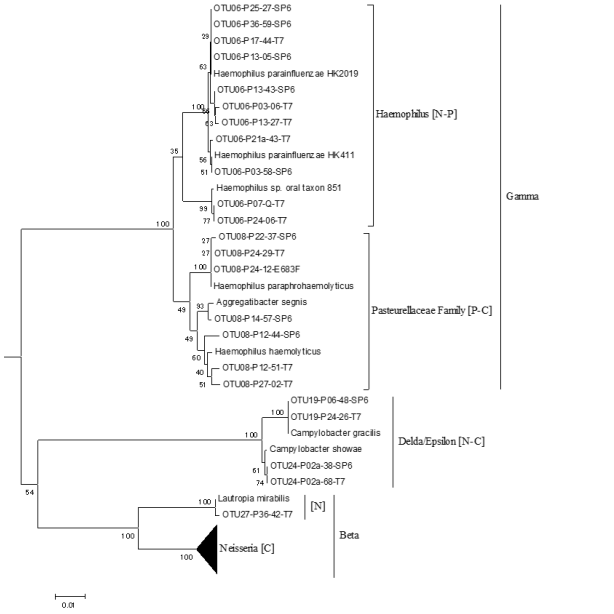
b. Clostridiales Order of the Firmicutes Phylum



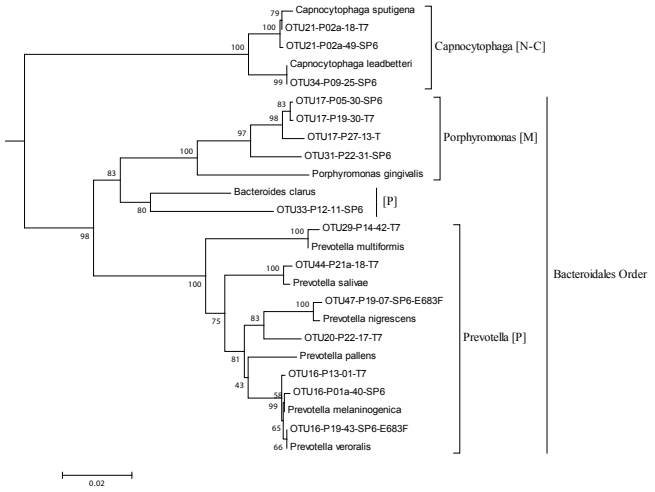
c. Actinobacteria Phylum



d. Fusobacteria Phylum



e. Proteobacteria Phylum



f. Bacteroidetes Phylum

FIG. 4: NJ tree was generated based on ClustalW alignment with 1000 bootstraps. The 16S reference sequences were selected based on top results with lowest E-values from HMP-DACC and NCBI 16S nucleotide databases. The sequences were clustered into five bacteria phyla which were Firmicutes (4a and b), Actinobacteria (4c), Fusobacteria (4d), Proteobacteria (4e) and Bacteroidetes (4f). Bacteria groups associated to different status of clinical samples such as normal were donated as [N], normal and OPMD as [N-P], OPMD as [P], OPMD and cancer as [P-C], cancer as [C], normal and cancer as [N-C] and all three groups as [M]

phyla with the exception of Fusobacteria. Firmicutes and Bacteroidetes were found to have more OPMD related bacteria groups while Proteobacteria was found to have more cancer related bacteria groups. Besides that, around half of the bacteria groups in Actinobacteria were found to be associated with both diseased conditions.

Community structure of oral microbiota

AMOVA results (Table 7) indicated that the significant genetic variations between all subject

TABLE 7: AMOVA based on V6-V9 regions of 16S rDNA which spans 751bp

Groups	Source of variations	SS	Df	MS	Fs	p-value
N-O-C	Among	0.1445	2	0.0723	3.0470	<0.05
	Within	0.4980	21	0.0237		
	Total	0.6426	23			
N-O	Among	0.0335	1	0.0335	1.4775	0.261
	Within	0.3403	15	0.0227		
	Total	0.3739	16			
O-C	Among	0.0414	1	0.0414	1.5798	0.227
	Within	0.3668	14	0.0262		
	Total	0.4082	15			
N-C	Among	0.1491	1	0.1491	6.7081	<0.05
	Within	0.2890	13	0.0222		
	Total	0.4381	14			

Abbreviations: N-O-C: normal, OPMD and cancer groups; N-O: normal and OPMD groups; O-C: OPMD and cancer groups; N-C: normal and cancer groups

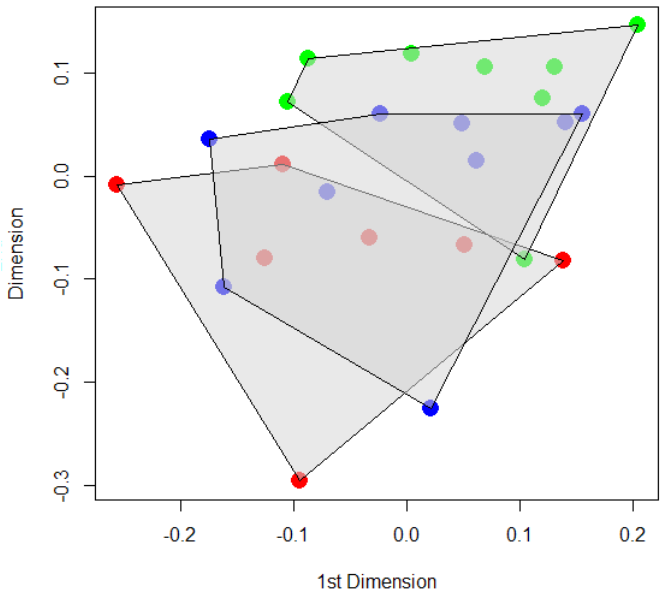


FIG. 5: Two Dimensional NMDS Plot with three groups of oral microbial communities

groups were due to the variations observed between normal and cancer groups. Although the genetic structure of bacteria diversity in the OPMD group was not significantly different from that of normal or cancer groups, two-dimensional NMDS plot showed that the bacteria community groups associated with OPMD subjects were sandwiched between that of normal and cancer subjects (Fig. 5). The 0.12 stress level indicated that the two-dimensional NMDS plot was a good representation of the similarities of the different oral microbiome communities. NMDS plot based on ethnicity and gender shows no distinct variations among different oral conditions (Figs. 6 & 7). This finding is in agreement with Nasidze *et al* (2009) in which no association between gender and age on oral microbial communities was mentioned.

DISCUSSION

The core human oral microbiome

This study was able to identify five reported major oral microbiome phyla.[41,42] The five bacterial phyla identified in this study (Fig. 2) were consistently reported as the main oral microbiome components in both healthy and diseased states such as oral cancer, halitosis and periodontitis.[15,31,33,43] With the exception of *Lachnoanaerobaculum*, bacterial genera that were identified in all the three oral conditions were also commonly reported in other cancer associated oral microbiome studies (Fig. 3).[15,24,25] However, *Lachnoanaerobaculum* was first isolated from human saliva and it was previously identified as *Clostridiales bacterium* by HMP-DACC and was also listed in the Human Oral Microbiome

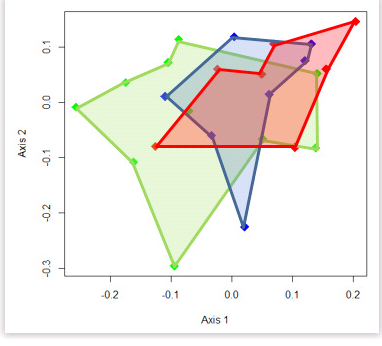


FIG. 6: Two Dimensional NMDS Plot of oral microbial communities with association of Chinese, Indian and Malay ethnicity

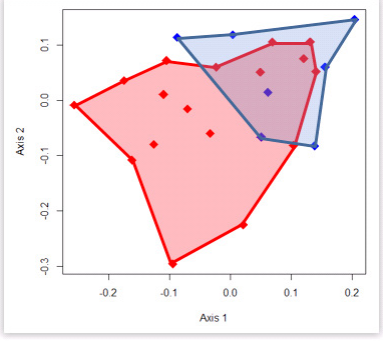


FIG. 7: Two Dimensional NMDS Plot of oral microbial communities with association of different genders

Database (HOMD).[44,45] This observation of consistent oral bacteria taxa suggests the presence of a common core oral microbiome in terms of genus and phylum levels across different oral states regardless of the detection methods such as culture[46], 16S cloned library and Next Generation Sequencing.[15,23-25,31,33,41-43]

The oral microbiome diversity

The higher effective species number of Shannon Index of both OPMD and cancer groups as compared to the normal group (Table 5) suggests that diseased oral conditions harbour more diverse oral microbiome community. This finding is in congruent with higher species diversity seen in diseased shallow and deep pockets of the oral cavities.[47]

The community structure of the normal and cancer associated oral microbiome was significantly different from each other (Table 7) which was in agreement with what previously reported where the DGGE fingerprint profiles of this two groups formed two distinct clusters.[24] In addition, this study also characterized the OPMD associated oral microbiome community structure that was not reported previously. The OPMD associated bacteria communities were shown to overlapped between the normal and cancer associated communities (Fig. 5) which conforms well to the OPMD definition, which is the transition stage between normal mucosa cells and OSCC.[48] This overlapping community membership could be the unique feature of OPMD associated oral microbiome and also the plausible reason of the insignificant differences found in the community structure between the OPMD and the other two groups (Table 7).

Oral microbiome membership associated with normal and OPMD and cancer oral cavities

The oral microflora diversity can be influenced by the host's dietary pattern and the sampling method of choice that could capture different sub-populations according to different targeted intraoral regions.[16,33] Besides, the community membership pattern can also be influenced by the immunocompromised states of the cancer patients. However, despite these variations, several oral bacterial species in this study were identified to have consistent association with their respective microenvironment (Table 6).

Eubacterium saphenum and *Selenomonas sputigena* is among the members of healthy core oral microbiome which is consistently identified in normal oral tissues.[15,23] On the other hand, *Camplylobacter showae* was more associated with cancer conditions since it was identified more in tumour tissues. It was reported that *C. showae* could coagulate with *Fusobacterium nucleatum* in some colon cancer cases.[49]

Several oral microbes were associated with diseased oral conditions as they were not only found in OPMD and cancer groups in this study but also isolated from oral tumour tissues from previous studies. *Catonella morbi* was detected at higher amount in OSCC tissue samples.[15] Besides that, *Rothia mucilaginosa* was found to be an emerging opportunistic pathogen due to frequent detection in immunocompromised hosts.[50] In addition, *R. dentocariosa* was associated with dental caries & periodontitis and is rarely involved in clinical infection.[51,52]

One of the major finding of this study is the identification of OPMD associated oral microbe, such as *Megasphaera micronuciformis*, *Prevotella melaninogenica* and *P. veroralis*, which could be potential biomarkers to detect this pre-cancerous transition stage. *M. micronuciformis* was first isolated from abscess samples and was found to be associated with immunocompromised HIV infected patients as an opportunist.[53,54] *P. melaninogenica* was found to be a potential salivary marker for early oral cancer detection besides being known as a periodontitis causal agent.[23,55] On the other hand, *P. veroralis* was only known to be isolated from the human oral cavity.[56] Nonetheless, it was reported that tumour tissues harboured higher amount of *Prevotella* species which suggested a possible strong relationship between oral cancer and certain *Prevotella* species such as *P. melaninogenica* and *P. veroralis*.[57]

OPMD associated oral microbes may appear as potential biomarkers of important medical value as these species allow the diagnosis of OPMD to be possible and may even permit early treatment interventions to occur before the start of oral cancer. Early detection and treatment of oral cancer would result in great improvements on the treatment outcome and the survival rate.[10,11] *M. micronuciformis* is potentially the best candidate for OPMD specific biomarker as it was detected only in the OPMD group in this study. Besides that, other oral microbes such as *P. melaninogenica*, *P. veroralis*, and *R. mucilaginosa* are also proposed to be possible biomarkers for early oral cancer detection as they were detected at higher abundance in OPMD oral conditions (Table 6). In addition, the consistent association of *Camplylobacter*

showae with cancerous oral conditions (Table 6) suggests that these oral microbes could be explored for other potential therapeutic values such as biomarkers for cancer prognosis or as vectors for targeted treatment.[58,59]

This study only identified patterns of oral microbiome variation between oral cancer and normal individuals without identifying specific confounding factors such as their immunocompromised and inflamed states. Subsequently, the exact role of reported disease associated microbes remains unknown. Nonetheless, the bacteria species identified as potential biomarkers for diagnostic, prognosis and therapeutic purposes in this study would require further confirmations from thorough investigation involving larger sample size of patient subjects, particularly patients who are diagnosed with OPMD and had transformed into cancer over a period of time.

Conclusion

A core human oral microbiome, in terms of common bacterial phyla and genera, was observed in the oral samples of all three types of subjects (normal, OPMD and oral cancer patients). The normal and cancer associated general oral microbiome were significantly distinct from each other while the community membership of the OPMD associated oral microbiome was found to overlap between the normal and cancer oral microbiota. Although microbiome diversity can be influenced by immunocompromised states and sampling methods, this study identified some consistent patterns that could have potential clinical applications. *M. micronuciformis* could be an important biomarker for early oral cancer detection due to its association only with the OPMD subjects. Besides that, other oral microbes with potential biomarker properties include *P. melaninogenica*, *P. veroralis* and *R. mucilaginosa* as they were identified at higher amount in OPMD groups. Other bacteria species such as *Camplylobacter showae* could have other potential medical values in tumour management such as cancer prognosis and treatment strategies since they were consistently associated to the cancer group. The identification of OPMD associated oral microbiome and OTUs provided a more inclusive view on the relationship between oral cancer, oral microbes and microbiome community structure. However, the diagnostic or therapeutic values of these bacterial species and groups require further validations with higher number of OPMD and oral cancer samples.

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