"EVALUATION OF BACTERIAL MICROFLORA ON THE SURFACE OF NORMAL ORAL MUCOSA, ORAL SQUAMOUS CELL CARCINOMA AND DEEPER TISSUES OF ORAL SQUAMOUS CELL CARCINOMA - A MICROBIOLOGICAL STUDY"

By

Dr. SHRADHA VAGARALI

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MASTER OF DENTAL SURGERY

In

ORAL & MAXILLOFACIAL PATHOLOGY & ORAL MICROBIOLOGY

(BRANCH - IV)

Under the Guidance of

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I have been given to understand that any research work I undertake for the purpose of dissertation, oral presentations or publications during my study course shall be property of the KAHER Vishwanath Katti Institute of Dental Science, Belagavi. Hence, I hereby declare that the name of department, institute and university shall be mentioned in my publications. The authorship shall be according to the guideline informed to me.

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This dissertation is dedicated to

My Parents

&

My Teachers

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" No one who achieves success does so without acknowledging the help of others"

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"It is in the roots, not the branches, that a tree's greatest strength lies"

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"Every journey comes to an end, but every end is a new beginning"

Dr. SHRADHA. VAGARALI

LIST OF ABBREVATIONS

SR. NO.	ABBREVATIONS	FULL FORM
1.	%	Percentage
2.	i.e.	That is
3.	No.	Number
4.	OSCC	Oral squamous cell carcinoma
5.	OS	Overall Survival
6.	MALT	Mucosa-associated lymphoid tissue
7.	ALDH-2	Aldehyde dehydrogenase-2
8.	IARC	International Agency for Research on Cancer
9.	HPV	Human Papilloma Virus
10.	AIDS	Acquired Immunodeficiency Syndrome
11.	GEC	Gingival epithelial cells
12.	JAK1/STAT	Janus Kinase/signal transducers and activators of transcription
13.	PI3K/AKT	Phosphatidylinositol 3-kinase/ protein kinase B
14.	BCL-2	B-cell lymphoma-2
15.	STAT3	Signal transducer and activator of transcription
16.	SOCS3	Suppressor of cytokine signalling 3
17.	LPS	Lipopolysaccharide
18.	CDKs	Cyclin-dependent kinases
19.	MMP-9	Matrix metalloproteinase-9
20.	ROS	Reactive oxygen species
21.	RNI	Reactive nitrogen intermediates

22.	TSI	Triple sugar iron
23.	MR	Methyl red
24.	VP	Vogues-Proskauer
25.	AST	Antibiotic sensitivity test
26.	CLSI	Clinical and Laboratory Standard Institute

ABSTRACT

AIM OF THE STUDY: Evaluation of bacterial microflora on the surface of normal oral mucosa, Oral Squamous Cell Carcinoma (OSCC) and in deeper tissues of OSCC using standard microbiological culture approach.

OBJECTIVES:

1. To evaluate the bacterial species, present on the surface of normal oral mucosa.
2. To evaluate the bacterial species, present on the surface and in deeper tissues of OSCC.
3. To compare the bacterial species, present on the surface of normal oral mucosa, surface of OSCC and in deeper tissues of OSCC.

MATERIAL AND METHODOLOGY: A total of 90 samples were taken for this study. 30 each from surface of normal oral mucosa (Control group), surface of OSCC (Sample A of study group) and deeper tissues of OSCC (Sample B of study group) using standard microbiological approach. Oral swabs were obtained from the surface of tumor site of oral squamous cell carcinoma after rinsing the oral cavity with normal saline. In the same patients, tissue specimen from within the tumor mass were obtained. The specimen of the size 0.2x0.2cm^2 and at a depth of minimum 1 cm from the surface of OSCC. Oral swabs from the surface of normal oral mucosa after rinsing the oral cavity with normal saline of subjects of matching age, sex and socio-economic strata with that of study group

RESULTS: The study group included a higher proportion of men, with a higher number of cases in the 51-60 year age range. Buccal mucosa being the most

commonly affected site in the present study. Most of the individuals with OSCC had a history of tobacco consumption. The surface of normal mucosa, OSCC and deeper tissues of OSCC showed presence of *Candida sp*., *Klebsiella pneumonia, Pneumococci, Staphylococcus aureus, coagulase negative staphylococcus species, Enterobacter, Micrococcus Peptostreptococcus, Pseudomonas and Streptococcus pyogens.* Anaerobic microorganisms such as *Bacteroides, Fusobacterium, Porphyromonas, and Prevotella* species were also found.

CONCLUSION: The study concluded that a group of asacchrolytic, periodontitis related microorganisms were found along with increased number of *Candida species* in deeper tissues of OSCC

KEYWORDS: Oral Squamous Cell Carcinoma, Microflora, Microbiota, Microbiome

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INTRODUCTION

Oral cancer ranks sixth among all types of cancer, with a greater prevalence in underdeveloped countries.[1] Squamous cell carcinomas originate from the oral mucosa and account for more than 90% of malignancies in the mouth.[2] Smoking, strong alcohol usage, and smokeless tobacco products are all linked to the development of oral cancer.

The fact that the above core risk factors can only account for 15% of oral malignancies has motivated researchers to explore into other probable risk factors. Viral infections, poor dental hygiene, and candida infection are all possible risk factors. *Candida's* link to certain types of leukoplakic lesions has long been known as a discrete risk factor.[3]

Helicobacter pylori is the most well-known bacterium linked to the development of cancer in humans, and it is classified as a class I carcinogen.[4] Peptic ulcer disease, chronic gastritis, gastric adenocarcinoma, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, these are all caused by *H. pylori*, which also causes intestinal metaplasia.[4] Simultaneously, evidence suggests that bacteria in the oral cavity have a role in cancer development.[5] Oral cancers, as well as tumors of the gastrointestinal tract, may be caused by such bacteria.

The association between the microbiome and oral cancer appears to be complex; particular microorganisms may have a role in tumor initiation and progression, or the entire microbial ecosystem's functional dysbiosis may contribute to the complex events of carcinogenesis and disease progression.[6,7] The effects of microbes on mammalian host gene expression have been found to be highly site-

specific. [8,9] According to research, there are significant differences in microbiome composition at most body sites that vary among countries and ethnicities, necessitating further investigation into geographic and cultural variations in the oral microbiome.[10,11] The bacteriome's involvement in a given environment is determined by its overall metabolic activity.[12,13] Changes in the tumor microenvironment caused by bacterial co-metabolism can have an impact on the host immune response as well as cancer therapeutics.[14,15]

Meanwhile, there has been a surge in interest in the putative links between microorganisms and various phases of cancer formation, and multiple methods by which bacteria and yeast may initiate or promote carcinogenesis are currently being researched. Many studies have connected acetaldehyde, to malignancies of digestive tract in both normal and deficient genotype of ALDH-2 (aldehyde dehydrogenase-2),[16-18] and there is mounting evidence that acetaldehyde, not alcohol, is to account for the carcinogenic effect of heavy drinking.[19-21]

Despite growing interest in the possible link between bacteria and various malignancies, the linkage between microorganisms and oral cancer has yet to be thoroughly investigated.

Patients with oral squamous cell carcinoma (OSCC) had significantly greater levels of particular bacteria in their saliva, according to new research.[22] This is of special importance since it has the potential to be used as a diagnostic tool for oral cancer prediction.

There has lately been evidence to suggest that the microflora associated with the surface of oral cancer differs from that of normal oral mucosa, which could play a role in disease progression.[23, 24]

There are very few studies which are done to evaluate bacterial species within deeper tissues of oral squamous cell carcinoma and no Indian research has been done so far in this regard. With this background we took up this study which aims at evaluation of bacterial species on the surface as well in deeper tissue of OSCC.

AIM OF THE STUDY:

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REVIEW OF LITERATURE

Oral cancer ranks among sixth most prevalent cancers in the world.[25] It ranks as one among the top three cancers in various parts of Asian-Pacific countries. [26] Squamous cell carcinomas of the pharyngeal & oral mucous membrane account for approximately 95 percent of all malignancies. [2] Cancers of the lip, other areas of the mouth, and the oropharynx are all examples of oral cancer.

As per the registry of International Agency for Research on Cancer (IARC) the global incidence of lip and oral cavity cancers is estimated to be 4 cases per 100,000 people. However, there is a wide range of reported incidences around the world, ranging from none to roughly 20 per 100,000 persons.[27]

India has the highest risk of oral cavity cancer among Asian countries. Their regional differences in disease incidence are most likely due to differences in societal customs. Chewing betel nut leaves wrapped with lime and tobacco, known as 'pan', results in prolonged contact of the carcinogens with the buccal mucosa and is regarded to be the primary cause of mouth cancer.[28] Tobacco smoking and alcohol drinking account for 74% of the population attributable risk. [29] Virus infections, in particular Human Papilloma Virus (HPV), explains why the younger people are more likely to develop cancer.[30] Genetic sensitivity stemming from variations in carcinogen metabolizing enzymes and DNA repair pathways, which happens frequently in the context of dietary micronutrient deficits, is another factor linked to oral carcinogenesis. [31-33] Excessive UV light, sulphur dioxide, pesticides, mists produced by strong inorganic acids, and the use of fossil fuels are other lesser-known risk factors. [34,35]

Oral infections in patients with conditions such as malignant tumors, leukemia, and AIDS were little understood until recently. Some of the most difficult oral infections to treat are in patients who have neoplastic diseases. One among them being oral cancer, whereby the patient is considered to be medically compromised, by virtue of his or her medical condition, is susceptible to infections or to serious complications. [36]

Microbes of the normal oral flora and non-oral microbes introduced from the outside may infect the tissues of the oral cavity. In general, the oral tissues show excellent resistance to infection. Diminished host resistance, in patients with diabetes, poor nutrition, malignant lymphoma, or primary or secondary immunodeficiency may lead to oral infections that are difficult to treat. Organisms that behave customarily as commensals then behave as pathogens and cause disease. [37]

DEFINITIONS

Microbiome: "The sum of microbes, their genetic information, and the environment in which they interact." [38]

Microbiota: "All living microbial organisms constituting the microbiome." [39]

Microflora: "Bacteria and microscopic algae and fungi, especially those living in a particular site or habitat." [40]

ORAL MICROFLORA IN HEALTH -

The oral cavity, which is one of the most highly colonized areas of the human body, has a bewildering assortment of microorganisms. This is owing to its unique anatomical characteristics which are found nowhere else in the body.

The major surfaces that habitat microflora are: [41]

- Tooth surfaces
- Buccal mucosa
- Dorsum of the tongue
- Supra & subgingival cervices
- Prosthesis and orthodontic appliances

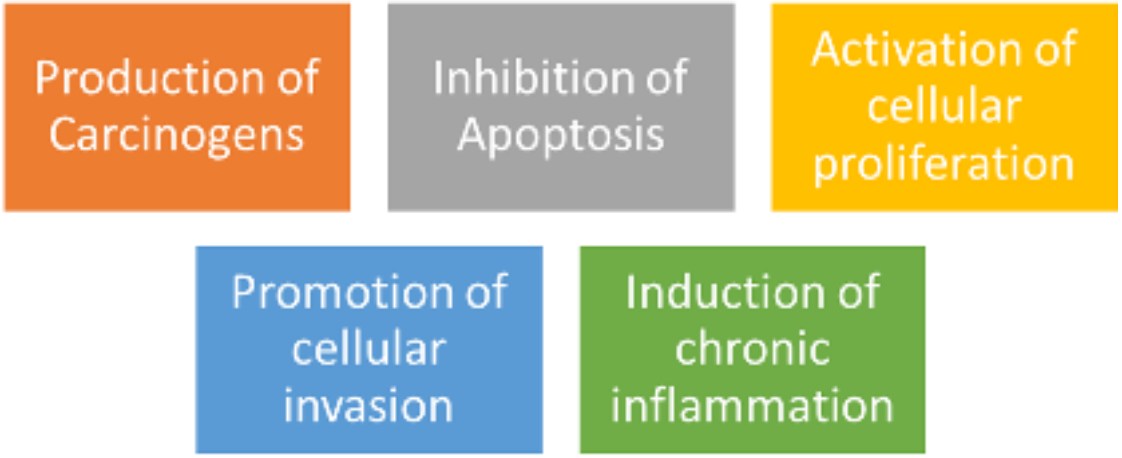
Under normal circumstances, bacteria, archaea, fungus, mycoplasmas, protozoa, and a viral flora maintain a balanced ecosystem. Bacteria are the most common occupants of the mouth with *Firmicutes, Bacillus, Proteobacteria*, and *Actinomycetes* being the most common. However, in disease, this ecological balance is disturbed leading to initiation and progression of various oral diseases. Diet, environment, medications, host genetics, and early microbial exposure are all factors to consider, which may affect the resident microflora. [36] The association between the microbiome and oral cancer appears to be complex; particular microorganisms may have a role in tumor initiation and progression, or the entire microbial ecosystem's functional dysbiosis may contribute to the complex events of carcinogenesis and disease progression.

MICROBIAL COMMUNITIES COMMONLY FOUND IN THE ORAL AND OROPHARYNX REGION [42]

Site	**Microorganism**
Tongue	*Veillonella atypica, Porphyromonas gingivalis, Selenomonas subspecies, Aggregatibacter species, Actinomycetemcomitans, Prevotella intermedia, Capnocytophaga, Enterococcus faecalis, Eikenella corrodens.*
Oropharynx	*Streptococcus pyogenes, Streptococcus pneumoniae, Hemophilus influenzae, Hemophilus parainfluenzae, Streptococcus mutans, Streptococcus salivarius, Streptococcus anginosus.*
Tooth Surface	*Streptococcus mutans, Actinomyces species, Eubacterium, Peptostreptococcus species.*
Tonsil	*Streptococcus viridans, Hemophilus influenzae, Neisseria species, Staphylococcus species.*
Gingival crevice	*Fusobacterium species, Prevotella species, Porphyromonas species, Streptococcus mitis, Streptococcus sanguinis, Propionibacterium acnes, Leptotrichia buccalis, Actinomyces odontolyticus, Veillonella parvula.*
Dental Plaque	*Actinomyces species, Rothia species, Microbacterium, Mycobacterium, Propionibacterium, Corynebacterium, Bifidobacterium.*

ORAL MICROFLORA IN CARCINOGENESIS

The possible mechanism by which bacteria induce carcinogenesis is as follows: [43]



Production of carcinogens:

Acetaldehyde, ethanol's first metabolite, has been found to be carcinogenic in mammalian tests and in vitro at relatively low concentrations, up to 100 mM(millimolar), which is the amount of saturation seen in saliva after moderate alcohol intake. [44-47] Many investigations have linked acetaldehyde to digestive tract cancers in both ALDH-2 defective and normal individuals with ALDH-2 gene, and there is strong evidence that acetaldehyde, not ethanol, causes carcinogenesis. [48-50] According to the International Agency for Research on Cancer, acetaldehyde is a Group 1 carcinogen in humans and is associated to alcohol consumption. Among other things, it can lead to sister chromatid substitutions, chromosomal aberrations, DNA hydroxylation, and epithelial hyperproliferation. Saliva contains mutagenic quantities of acetaldehyde during and after alcohol consumption, as well as smoking. Acetaldehyde levels in normal human saliva, on the other hand, are undetectable.[51-55]

Inhibition of Apoptosis:

P. gingivalis suppresses drug-induced apoptosis in gingival epithelial cells in primary cultures (GECs), which appears to be mediated by a number of mechanisms.[56] *P. gingivalis* stimulates JAK1/STAT3 and PI3K/Akt signalling. At the mitochondrial membrane, the activity of the proapoptotic BCL-2-associated death promoter (BAD) is blocked, resulting in an increased BCL2 (antiapoptotic): BAX (proapoptotic) ratio, which prevents the release of the apoptosis effector Cytochrome C.[57-59] *P. gingivalis* has also been shown to upregulate microRNA-203 in GECs, which increases the activity of STAT3 (signal transducer and activator of transcription) and decreases apoptosis by downregulating SOCS3 (suppressor of cytokine signalling 3).[60]

Activation of cellular proliferation:

P. gingivalis accelerates the creation of GECs in the S and G2 phases of the cell cycle, as well as activation (phosphorylation) of cyclin-dependent kinases (CDKs) and decreased expression of the tumour suppressor p53 gene, via upregulating cyclins. Lipopolysaccharide (LPS) from *P. gingivalis* might have a similar impact to bacterial lipopolysaccharide (LPS), which has been found to dysregulate p53.[61,62] *P. gingivalis* may contribute to a proliferative phenotype in GECs through a gingipain-dependent proteolytic process that activates beta-catenin.[63] *F. nucleatum* also promotes cell growth. Infection with *F. nucleatum* also causes overexpression of 12 kinases in human epithelial cells, the majority of which are involved in cell proliferation, survival, and DNA repair.[64]

Promotion of cellular invasion:

P. gingivalis and *F. nucleatum* promote cellular invasion. The activation of the ERK1/2-ETS1, p38/HSP27, and PAR/NF-kB pathways by *P. gingivalis* infection was demonstrated to increase production of pro-matrix metalloproteinase-9 (pro-MMP-9) in an OSCC cell line, with MMP-9 being cleaved into its active proenzyme by gingipains, boosting cellular invasion. *P. gingivalis* exposure can also increase OSCC cell invasiveness by promoting EMT, stemness acquisition, and increased MMP-1 and MMP-10 production.[65,66] Infection of human epithelial cells with *F. nucleatum* increases MMP-13 (collagenase 3) production and promotes cellular migration via activating mitogen activated protein kinase p38, likely via the Etk/BMX pathway.[64]

Induction of chronic inflammation:

Chronic inflammation is crucial in all phases of cancer development, including induction, progression, invasion, and metastasis, and is triggered by infections or environmental exposures.[67] Reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), and cytokines, which are produced by inflammatory cells, are hypothesized to contribute to cancer formation by inducing mutations, genomic instability, and epigenetic alterations. Within premalignant cells, inflammatory cytokines activate important transcription factors like STAT3 and NF-kB, promoting pro-malignant activities including proliferation, angiogenesis, invasion, and metastasis, which in turn maintains the microenvironment and leads to tumour growth.[67,68] *F. nucleatum* species generate high quantities of cytokines, which have been demonstrated to have pro-inflammatory potential.[69,70]

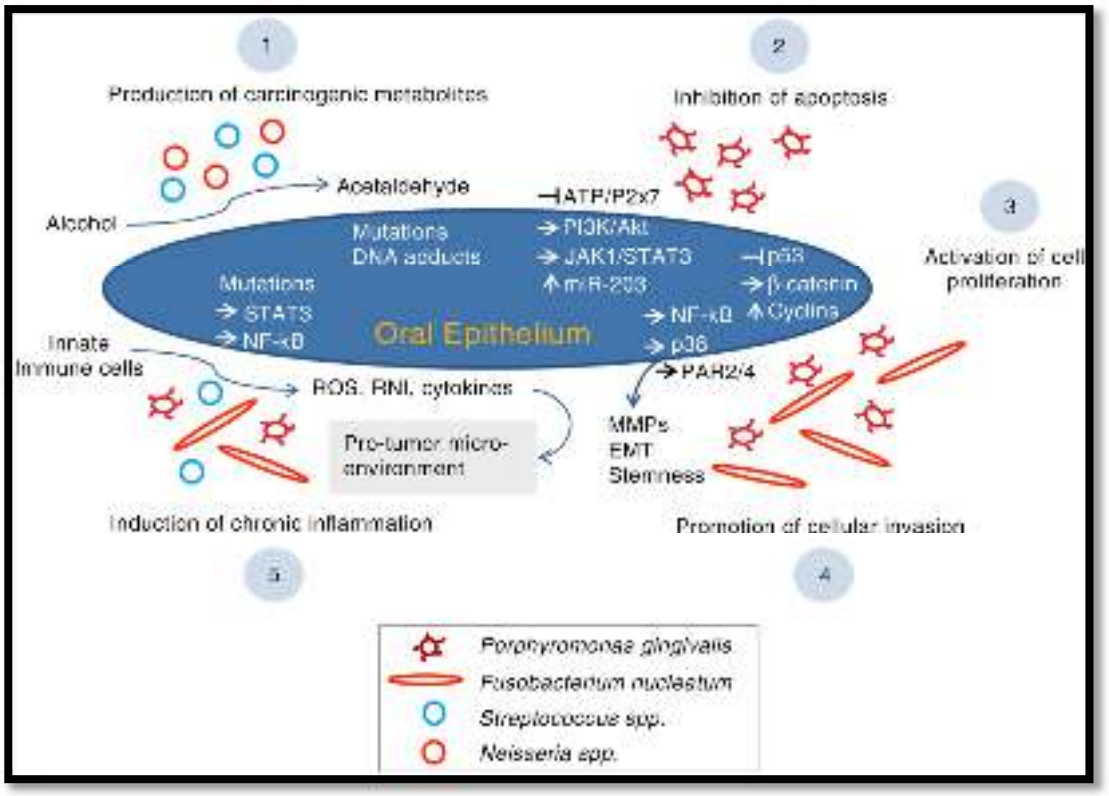


Figure 1: Role of Oral microflora in carcinogenesis. [43]

ORAL MICROFLORA IN OSCC

Nagy et al., [71] conducted the first study in the year **1998**, which aimed at investigating contents of the biofilm seen on the surface of OSCC. The central surface of the lesions was swabbed from 21 patients, with a 52.8-year-old median age as control, adjacent healthy mucosa was swabbed, before any antibiotic treatment or any resection of the tumor. Brain heart infusion (BHI) broth was the transport media used, the samples were cultured using aerobic, anaerobic and selective media. The tumor sites showed a significantly higher anaerobic colony forming units than the healthy controls. The results were in accordance to the aerobes at both, the tumorous site as well as the healthy mucosa. Anaerobes such as *Veillonella species, Fusobacterium species, Porphyromonas species, Actinomyces* & *Clostridium species*, and aerobes such as *Hemophilus species, Enterobacteriaceae* and *Streptococcus species.* were

found in the tumor sites. 8 among the 21 tumor sites also showed presence of *Candida albicans*. The study concluded that the surface of OSCC harbors significantly increased number of aerobes & anaerobes when compared to heathy surfaces of the same patient.

Helicobacter pylori has been linked to gastric cancers, not simply stomach ulcers, according to research. There have recently been reports that the DNA sequence of *Streptococcus anginosus (S. anginosus)* was discovered in DNA extracted samples from esophageal tumours. As a result, **Tateda et al., 2000**, [72] looked into the involvement of *S. anginosus* in the development of head and neck malignancies. PCR analysis using 16S ribosomal DNA-specific primers and Southern blot analysis were used to examine 217 DNA samples. The gingival smears of the patients were subjected to bacterial culture for the identification of *S. anginosus* in the oral and pharyngeal cavities, as well as PCR analysis. PCR analysis revealed the DNA sequence of *S. anginosus* in all samples from head and neck malignancies. Southern blot analysis revealed positive bands in 33% of the samples. According to the findings, *S. anginosus* infection may have a role in the development of head and neck squamous cell carcinoma.

Morita et al., 2003, [73] did another investigation on the involvement of *Streptococcus anginosus (S. anginosus)* in oral and esophageal cancer. They used real-time PCR as a highly specific quantification approach for *S. anginosus*. An experiment was also performed to see if *S. anginosus* was linked to oral cancer tissue. Only by employing a precise quantification technique could varying degrees of severity of *S. anginosus* infection be revealed. *S. anginosus* levels were found to be higher in esophageal cancer samples than in oral cancer samples when 10ng of genomic DNA was

extracted from cancer tissues. The DNA of *S. anginosus* was found to be ten times greater in esophageal cancer tissue than in oral cancer tissue. These findings showed that *S. anginosus* is more likely to be linked to esophageal carcinoma than to mouth cancer.

In contrast to the previous study, **Sasaki et al., 2005**, [74] investigated the function of *S. anginosus* in oral malignancies and proposed that *S. anginosus* infections, which may be seen in OSCC and dental plaque, operate as a dominating reservoir of *S. anginosus.* They used 46 OSCC tissue specimens, 3 precancer tissue specimens, and dental plaque samples to perform a species-specific Polymerase chain reaction (PCR) experiment. The genotype of clinical isolates from OSCC and dental plaque samples was determined using pulsed-field gel electrophoresis (PFGE), and it was discovered that the genotype of *S. anginosus* isolated from cancer tissue was comparable to that of dental plaque from the same individuals. The study also discovered that *S. anginosus* was mostly found in dental plaque and not in OSCC patients' saliva.

Till date, only two studies have been published in the literature which assessed viable bacteria in deeper tissue of OSCC. A preliminary study was done by **Hooper et al., in 2006**, [23] to identify bacterial species within OSCC tissue. The 20 tissue samples were obtained during surgery, approximately of the size 1 cubic meter. Another, 19 samples from the superficial surface of tumor and 12 from non-tumorous control 5-cm away from the primary tumor. Immersion in betadine and phosphate buffered saline was done to avoid surface contamination. The samples were then processed for aerobic and anaerobic culture using standard microbiological approach. Further 16S rRNA sequencing was also done to identify isolates. The study identified and isolated

a variety of bacterial taxa along with a few novel species. Saccharolytic, acid-resistant species were seen predominantly, due to unsuccessful surface decontamination, it showed the presence of bacteria within the deeper tissue.

As an extension of the above study **Hooper et al., 2007;** [24] performed a molecular analysis using Fluorescent in situ hybridization. The DNA from macerated tissue specimen was extracted. Further, 16S rRNA sequencing was done with three sets of primer for Spirochaetes, Bacteroidetes, and the domain bacteria. As many as 70 taxa were identified in total: 52 from different phyla of tumorous tissue, 37 from non-tumorous tissue. There was apparent change in the composition of microbiotas in tumor and tumor-free specimens.

Mager et al., 2005; [75] attempted to estimate oral bacteria salivary counts in OSCC patients and OSCC-free controls. The DNA hybridization method was used to evaluate the unstimulated saliva of 229 OSCC-free and 45 OSCC participants. *Capnocytophaga gingivalis, Prevotella melaninogenica,* and *Streptococcus mitis* were three of the 40 species studied that exhibited higher amounts in saliva. These species also had a sensitivity of 80% and a specificity of 83 % in predicting cancer incidences. *Capnocytophaga gingivalis, Prevotella melaninogenica*, and *Streptococcus mitis* may be relevant as diagnostic markers of OSCC, according to the study.

Various culture-independent studies done by several authors, [75-79] analyzed the oral microflora in OSCC using 16S rRNA sequencing in variable gene fragments ranging from V1-V5 region. Saliva, oral rinse, oral swab and tissue were the commonly used samples. Various organisms such as *C. gingivalis, P. melaninogenica, Streptococcus mitis, F. nucleatum, P. intermedia, A. segnis, P.*

stomatis, and C. morbi were isolated. Oral Squamous Cell Carcinoma was associated with higher abundances of bacteria.

ORAL MICROFLORA IN ORAL POTENTIALLY MALIGNANT DISORDERS (OPMD'S)

The sample types used in the two investigations reporting the microbiome associated with oral leukoplakia were different (swab and saliva), making a comparison difficult. The genera found to be associated with OSCC in these studies differed; *Haemophilus* was found to be significantly enriched in oral leukoplakia in the study [80], and *Fusobacterium, Leptotrichia,* and *Campylobacterin* were found to be significantly enriched in another study. [81]

There were four investigations on oral lichen planus [82-86] that employed diverse sample types to elucidate the bacteriome. **Kragelund et al., 2018,** [84] did not include a control group in their investigation, but they did characterise the mycobiome. In oral lichen planus, no one genus or species among the dominating taxa was considerably raised.

ROLE OF FUSOBACTERIUM NUCLEATUM AND PORPHYROMONAS GINGIVALIS IN PROGNOSIS OF OSCC

Chen et al. in 2021., [87] studied the association of *Porphyromonas gingivalis* (*P. gingivalis*) and OSCC along with its prognostic value. They noted the abundance of *P. gingivalis* in saliva and OSCC tissues which showed that overabundance of *P. gingivalis* in saliva showed a favorable prognostic outcome in OSCC.

OSCC, hepatocellular carcinoma, and esophageal carcinoma were all linked to *P. gingivalis.*[88-91] Furthermore, in esophageal squamous cell carcinoma, individuals with a high level of *P. gingivalis* had the poorest prognosis.

Evidence of a link between *P. gingivalis* and OSCC has been growing recently. *P. gingivalis* stimulates immortalized oral keratinocytes resulting in a more aggressive malignant profile phenotype, resulting to improved tumour characteristics.[92] In OSCC patients, the serum immunoglobulin G antibody against *P. gingivalis* is greater than in non-OSCC patients.[93] *P. gingivalis* elevated the size and frequency of carcinomas to facilitate the spread of oral carcinomas.[94] Previous research examined the amount of *P. gingivalis* in patients' saliva and discovered that patients with moderate to poor differentiation, overall clinical stages III and IV, lymph node metastases, and shorter overall survival had *P. gingivalis* involvement.[95,96] Early-stage patients have a one-year survival rate of 90–95 percent, while advanced-stage patients have a one-year survival rate of 65–70 percent.[97]

The overabundance of *P. gingivalis* in saliva from OSCC patients was confirmed in this investigation as compared to OSCC-free participants, and the overabundance of *P. gingivalis* in saliva was enhanced with the advanced clinical stage. The existence of fimbriae, which play an important part in adhesion, colonization, and invasion, is thought to be one of the most essential virulence factors in *P. gingivalis*.[98] The majority of the research looked at how fimA genotypes were distributed in periodontitis. FimA genotypes have been linked to more severe infections and produce inflammations.[99,100] The frequency of fimA in OSCC however is unclear. The present study, showed the distribution of fimA subtypes in saliva. Least amount of genotype V was noted in this study, possibly due to is low prevalence

(0-20%). [101] ***Mendoza et al., in 2019,*** published a systematic review which investigated the role of *P. gingivalis* in development of OSCC. *P. gingivalis* plays a role in development of OSCC through epithelial-mesenchymal interaction [102-108] neoplastic cell proliferation [101,104,106,107,] and tumor cell invasiveness. [108-112]. However, the role of this bacterium in the prognosis of the disease is yet to be explored further.

The relationship of *F. nucleatum* species with the tumor and its clinicopathological characteristics, recurrence and overall survival (OS) of two distinct groups with OSCC as well as genes linked to the immune system were examined in 2021 by **Cindy Neuzillet et al.**[113]

This mechanism has been explained by decreased RNA concentrations of M2 macrophages, fibroblasts, CD^4 cells, TLR4 , TNFRSF9, QX40 ligand and high amounts of TNFSF9 and interleukin-1 that were found in *F.nucleatum* loads.[114]

F.nucleatum is linked with a "permissive" tumor milieu which show resistance for pro inflammatory signals, have minimal signaling of TLR4 and have decreased M2 intake results in amplified clinical outcomes. [115,116]

MATERIALS AND METHODOLOGY:

The present clinical study comprised of total 90 samples. The individuals were selected amongst those coming to Out-patient department of:

1. KLE V K Institute of Dental Sciences, Belagavi
2. KLE's Belgaum Cancer Hospital and Research Centre, Belagavi
3. KLE's Dr. Prabhakar Kore Hospital and Research Centre, Belagavi
4. Karnataka Cancer Therapy and Research Institute Navanagar, Hubli

CASE SELECTION:

A total of 30 clinically diagnosed and histopathologically verified cases of OSCC were included in this investigation, the control group consisted of 30 individuals with apparently normal oral mucosa. A detailed case history was recorded. (Annexure-1)

SAMPLE SIZE ESTIMATION:

The below equation was used to determine sample size:

$$n = \frac{2pq(z_\alpha + z_\beta)^2}{d^2}$$

$p = \frac{p_1 + p_2}{z}$, $q = 100 - p$, $d = p_1 - p_2 = 25\%$ $Z_\alpha = 1.96$ at 5% α error

$Z_\beta = 0.842$ at 70% β error

n=42

Due to COVID-19 pandemic, in consultation with the biostatistician, the same size was set to 30. (Biostatistician certificate- Annexure-II)

Hence, **Sample size=30**

i.e., **Study group- Sample A(Oral swab)=30**

Study group- Sample B (Deeper Tissue)= 30

Control group= 30

Total sample size: 90

INCLUSION CRITERIA:

For study group(OSCC group):

- Cases of oral squamous cell carcinoma that were clinically diagnosed and histopathologically confirmed.

For control group:

- Patients with apparently normal oral mucosa without OSCC, with matching age, sex and socioeconomic status as that of the study group.

EXCLUSION CRITERIA:

For study group(OSCC group):

- Patients with previous history of treatment for OSCC
- Patients undergoing chemotherapy or radiation therapy
- Patients under long term antibiotic treatment
- Patients suffering from systemic disorders such as diabetes.

For control group:

- Patients with any frank oral lesion
- Patients with any other gingival and periodontal lesions
- Patients under long term antibiotic treatment
- Patient having tobacco habits
- Patients having systemic diseases such as Diabetes mellitus.

Ethical approval and informed consent were obtained from all the patients prior to sample collection. (Annexure III & annexure IV)

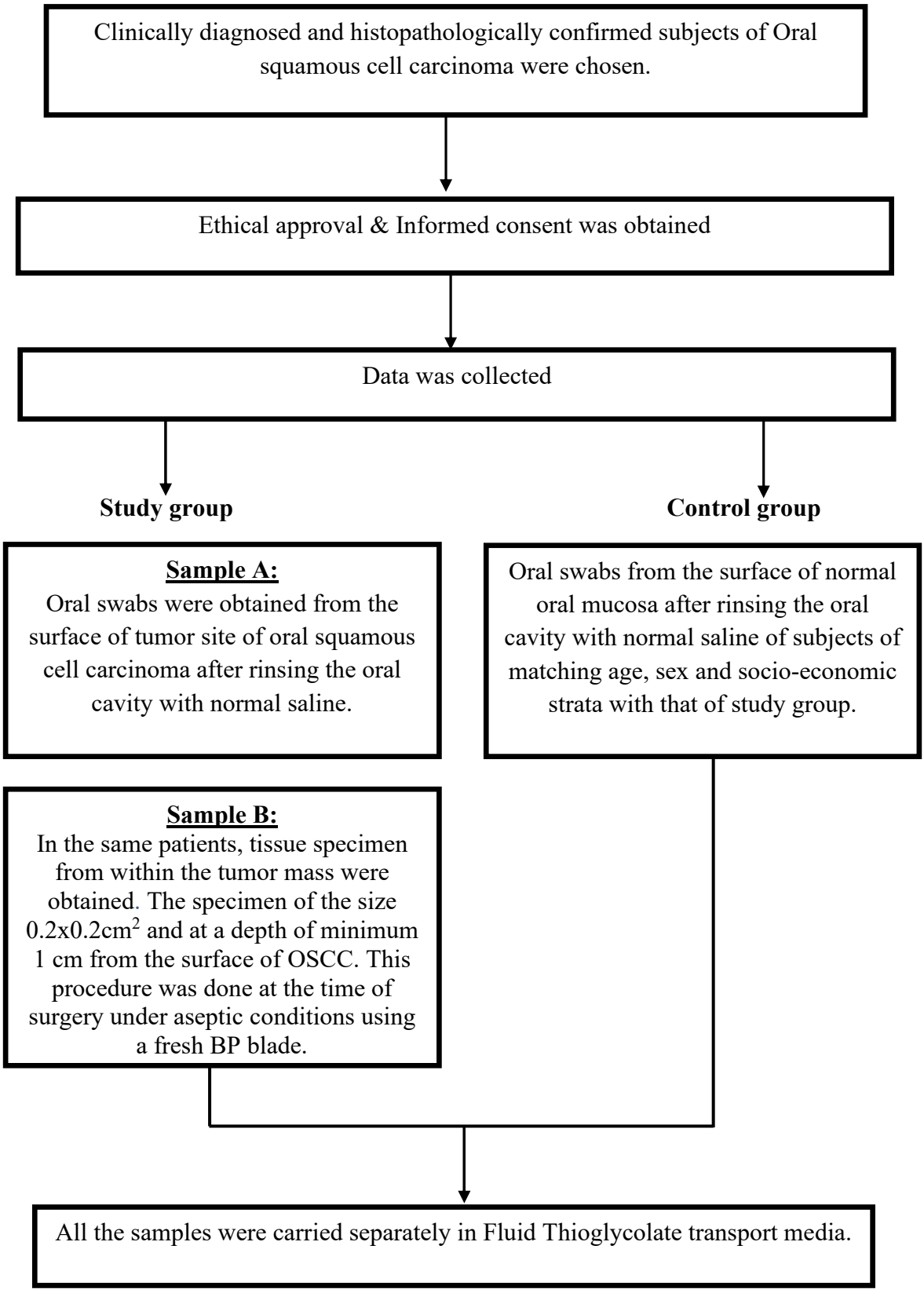
Patient information sheet was given to each patient- Annexure-V

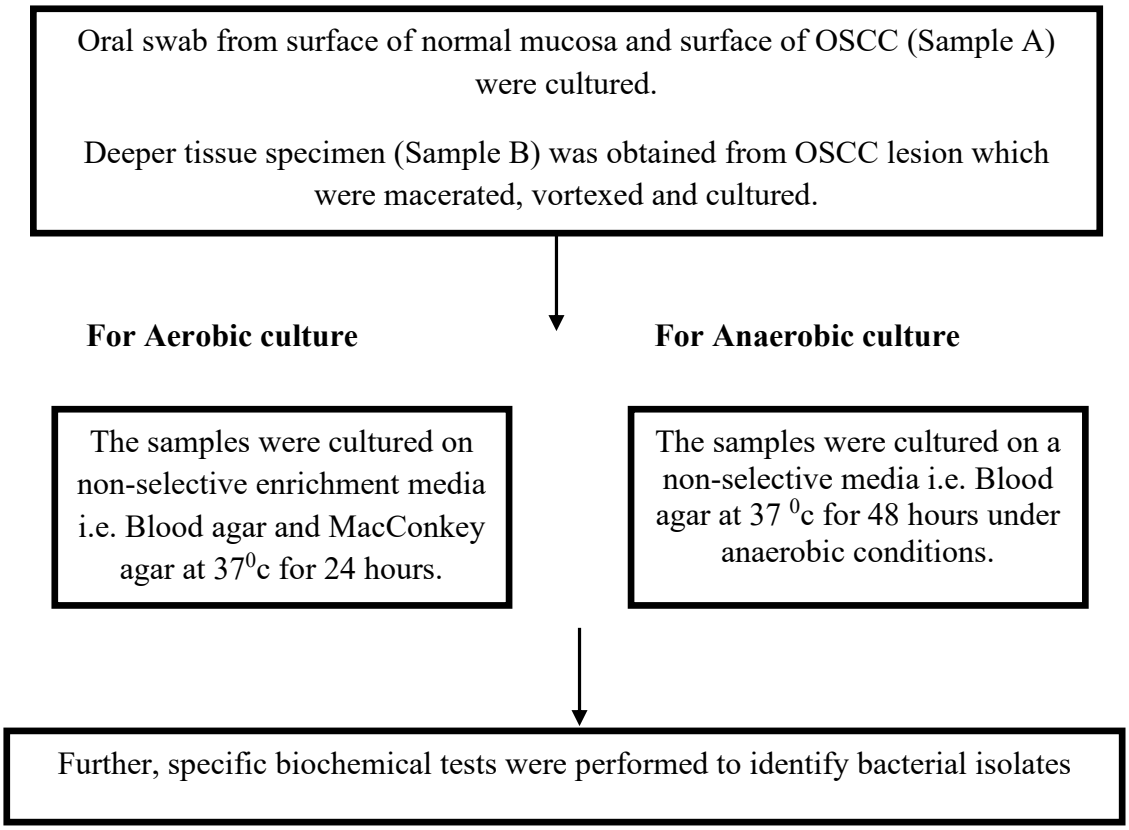
MATERIALS AND ARMAMENTARIUM:

Materials: Brucella blood agar, MacConkey agar, Nutrient agar, Fluid thioglycolate medium, Peptone water media, Mannitol motility test medium, Urea agar base, Simmons citrate agar, Triple sugar iron agar, Anaerobic gas packs, Sugars such as : glucose, fructose, maltose, sucrose, galactose and lactose

Armamentarium: Petri dish, concealed swabs, inoculating loops, Anaerobic gas pak-jar.

METHODOLOGY WITH FLOWCHART:





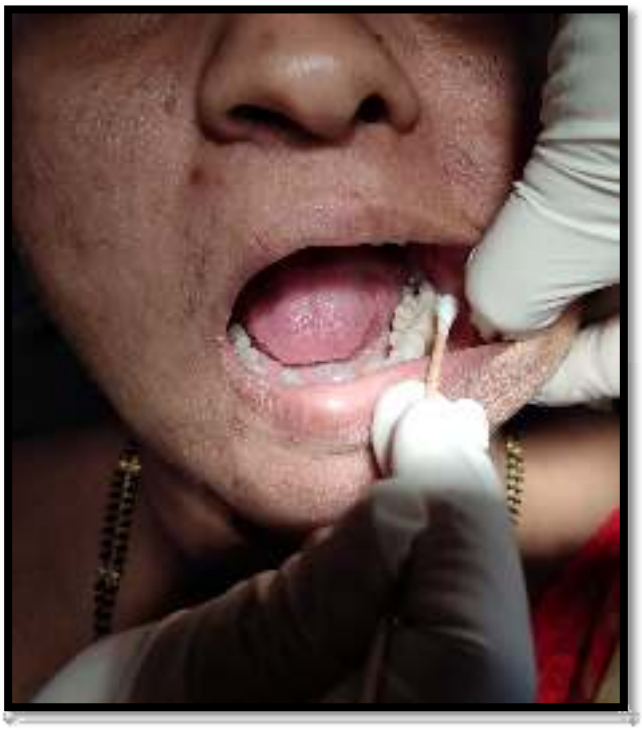
(Overview of sample processing – aerobically & anaerobically- Annexure- VI)

(Preparation of Thioglycolate broth, Brucella blood agar, MacConkey agar & media for biochemical reactions- Annexure-VII)

STATISTICAL ANALYSIS:

The Chi-square test and descriptive statistics were used to analyse the data statistically.

Photograph.1: **Patient was asked to rinse the oral cavity with normal saline.**



Photograph.2: **Swabs were taken from the surface of normal oral mucosa (control group) and the surface of oral squamous cell carcinoma.(Sample A of OSCC group)**

Photograph. 3: **Deeper tissue specimen of OSCC (Sample B) was taken from within the tumor mass. The tissue was taken at a depth of minimum 1cm^2 from the surface of OSCC.**

Photograph.4 & 5: **All the samples were transported in Fluid Thioglycolate media in a sterile test tube and container.**

Photograph.6: **The samples were streaked on Brucella Blood agar with Vitamin K and Hemin for aerobic and anaerobic culture.**

Photograph.7: **The samples were also streaked on MacConkey agar plates to identify lactose fermenting and non-lactose fermenting colonies.**

Photograph.8: **Anaerobic Gas-Pak jar system was used to create an oxygen-free environment for the growth of anaerobic microorganisms.**

RESULTS

Our study compared the type of bacteria present on:

1. Surface of normal oral mucosa- **CONTROL GROUP**
2. Surface of OSCC - Sample A
3. Deeper tissue of OSCC – Sample B

} **OSCC GROUP**

Each group consisted of 30 samples each, in total, 90 samples were included in the present study.

Table.1: The following table shows frequency and percentage of Age distribution in the OSCC group.

Age group (Years)	OSCC group- Frequency	OSCC group- Percentage (%)
21-30	3	10.00
31-40	2	6.67
41-50	5	16.67
51-60	16	53.33
>=61	4	13.33
Total	30	100.00

Inference: Table 1 shows more cases in OSCC group were in the age range of 51-60 years.

Table.2: The following table shows frequency and percentage of Gender distribution in the OSCC group.

Gender	OSCC group- Frequency	OSCC group –Percentage (%)
Male	24	80.00
Female	6	20.00
Total	30	100.00

Inference: Table 2 shows maximum number of males in the OSCC group, with a male is to female ratio of 4:1.

Table.3: The following table shows frequency and percentage of distribution of cases by site in the OSCC group.

Site	OSCC group – Frequency	OSCC group – Percentage (%)
Alveolus	4	13.33
Buccal mucosa	13	43.33
Buccal mucosa and tongue	1	3.33
Floor of the mouth	2	6.66
Gingivobuccal sulcus	4	13.33
Tongue	6	20
Total	30	100

Inference: Table 3 shows that in the OSCC group buccal mucosa was the predominantly affected site.

Table.4: The following table shows frequency and percentage of individuals with and without habit history in the OSCC group.

Habit history	OSCC group- Frequency	OSCC group – Percentage (%)
Present	22	73.33
Absent	8	26.77
Total	30	100

Inference: Table 4 shows that in the OSCC group, maximum number of individuals had history of consuming tobacco in any form.

Table.5: The following table shows frequency and percentage of Age distribution of cases in the control group

Age groups (Years)	Control group- Frequency	Control group – Percentage (%)
21-30	2	6.67
31-40	9	30.00
41-50	9	30.00
51-60	6	20.00
>=61	4	13.33
Total	30	100.00

Inference: Table 5 shows a greater number of cases were in age range of 31- 40 and 41-50 years.

Table.6 : The following table shows frequency and percentage of Gender distribution in the control group.

Gender	Control group- Frequency	Control group – Percentage (%)
Male	17	56.67
Female	13	43.33
Total	30	100.00

Inference: Table 6 shows, a slightly higher number of cases were males in control group.

All the cases in control group, were taken from the gingivobuccal sulcus area, hence no table for site distribution in the control group was made.

The cases with no habit history were chosen for the control group, to avoid habit as a confounding factor.

Table.7: The following table shows comparison of frequency and percentage of age distribution in the OSCC and Control group.

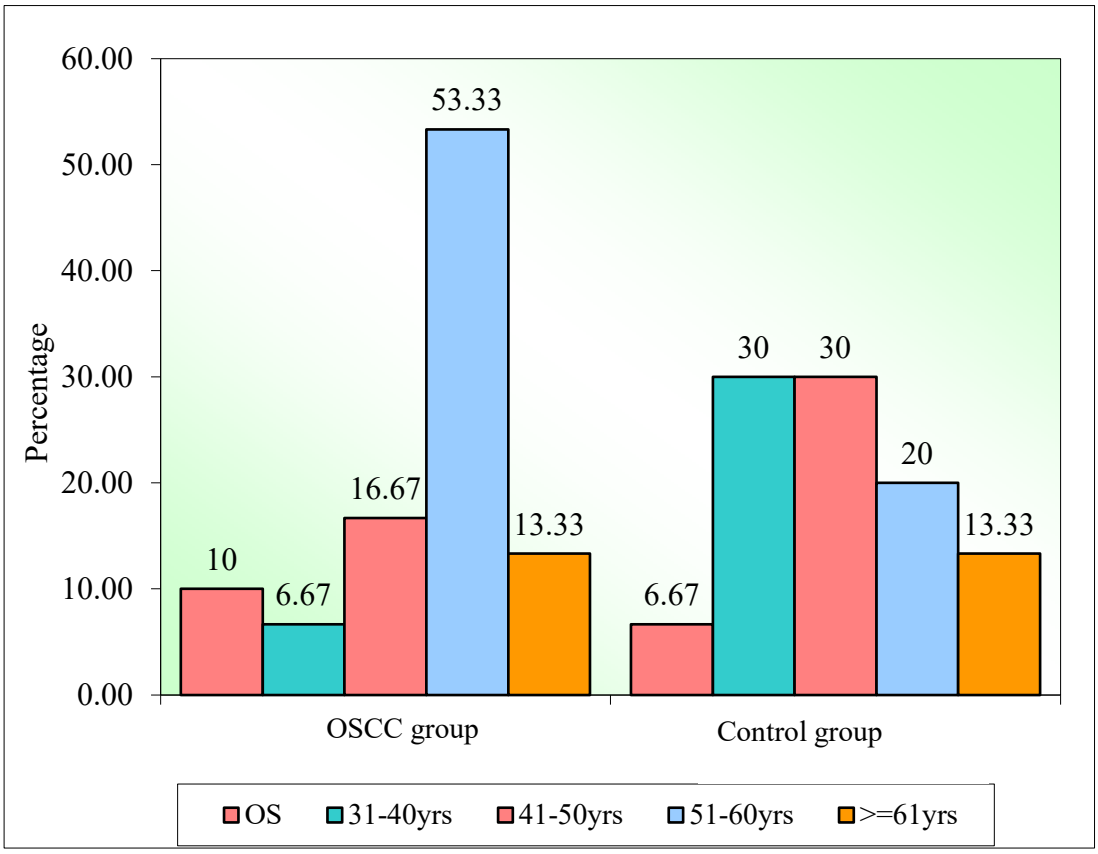
Age groups (Years)	OSCC group- Frequency	OSCC group- Percentage (%)	Control group- Frequency	Control group- Percentage (%)	Total- Frequency	Total- Percentage (%)
21-30	3	10.00	2	6.67	5	8.33
31-40	2	6.67	9	30.00	11	18.33
41-50	5	16.67	9	30.00	14	23.33
51-60	16	53.33	6	20.00	22	36.67
>=61	4	13.33	4	13.33	8	13.33
Total	30	100.00	30	100.00	60	100.00
Mean	51.10		45.67		48.38	
SD	10.88		11.56		11.46	
Chi-square=10.3430 p = 0.0350*						

*p<0.05

Inference: Table 7 depicts the comparison of frequency & percentage in the OSCC group and control group. The maximum number of cases belonged to the age range from 51-60 years in the OSCC group and 31-40 & 41-50 years in the control group. Mean age of 51.10 with standard deviation of 10.88 was seen in OSCC group and a mean age of 45.67 with standard deviation of 11.56 in the control group. To compare

these two groups, a Chi-square test was used, yielding a p value of 0.0350 i.e, p value <0.05, showing statistical significance.

Figure.2: Bar graphs depicting percentage of age distribution in OSCC and Control group.



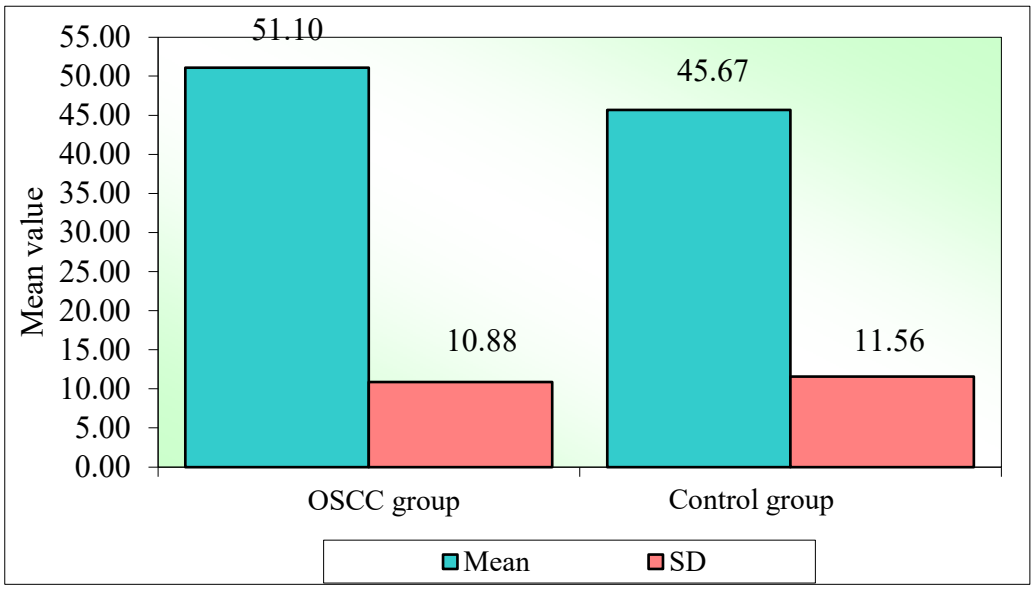
Inference: The bar graph shows more cases were seen in OSCC group in the age range of 51-60 years and most of the cases in control were in age group of 31-40 and 41-50 years.

Table.8: The following table shows comparison of mean age in both the groups using t-test.

Groups	n	Mean	SD	SE	t-value	P-value
OSCC group	30	51.10	10.88	1.99	1.8748	0.0659
Control group	30	45.67	11.56	2.11		

Inference: The table shows a comparison of mean age in both the groups. The mean age and Standard deviation (SD) in OSCC were 51.10 and 10.88 respectively, and that in the control group were 45.67 and 11.56 respectively. Standard error of 1.99 and 2.11 was seen in OSCC and control group respectively. P value is 0.0659 ($p > 0.05$), hence showing no statistically significant change in the mean age of both the groups.

Figure.3: Bar graph representing mean age and standard deviation in both the groups.



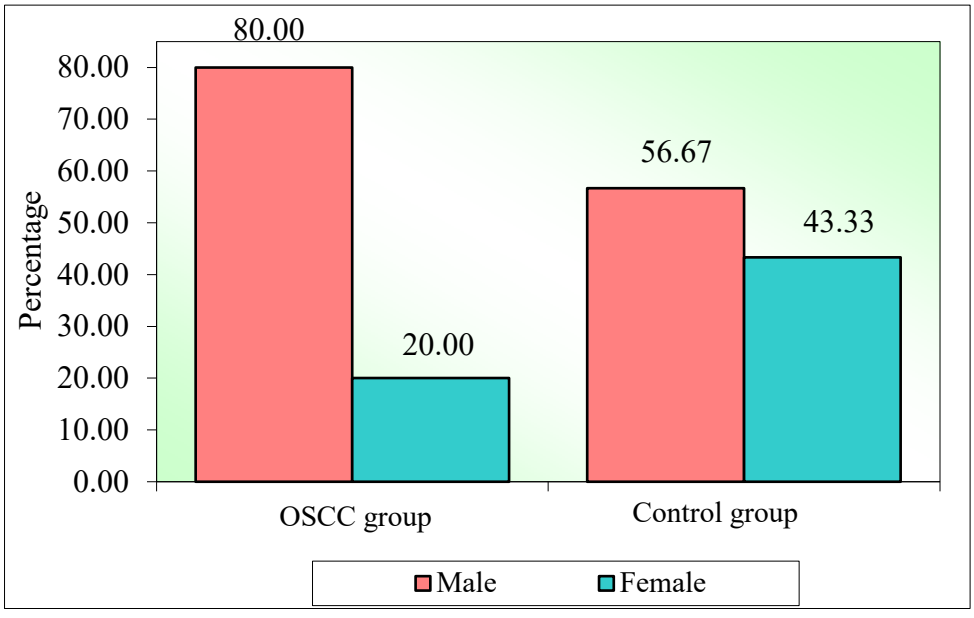
Inference: Bar graph depicting the mean age of 51.10 and standard deviation of 10.88 in OSCC group. The mean age in control group was 45.67 and standard deviation of 11.56.

Table.9: The following table shows comparison of frequency and percentage of gender distribution in both the groups.

Gender	OSCC group - Frequency	OSCC group - Percentage (%)	Control group - Frequency	Control group – Percentage (%)	Total - Frequency	Total – Percentage (%)
Male	24	80.00	17	56.67	41	68.33
Female	6	20.00	13	43.33	19	31.67
Total	30	100.00	30	100.00	60	100.00
Chi-square=3.7740 p = 0.0520						

Inference: Table 9 shows the comparison of gender distribution in the two groups by. A higher number of the cases were seen among males in the OSCC group and control group. A Chi-square test was performed, yielding a p value of 0.0520.

Figure.4: Bar graph depicting the gender distribution of cases in both the groups



Inference: Bar graph shows a greater number of cases among males in both, the OSCC and control group.

Table.10: The following table shows frequency of isolated aerobic organisms in three groups

Isolated organism – Aerobic culture	Surface of normal oral mucosa (Control group)	Surface of OSCC (OSCC group)	Deeper tissue of OSCC (OSCC group)	Total
Candida species	6	1	5	12
Coagulase negative staphylococcus	1	0	0	1
Enterobacter species	1	0	0	1
Escherichia coli	0	1	0	1
Klebsiella pneumonia	6	3	1	10
Oral commensals	9	4	1	14
Micrococcus species	1	0	0	1
Peptostreptococcus species	1	1	0	2
Pneumococci species	5	7	2	14
Proteus species	0	1	1	2
Pseudomonas species	1	7	2	10
Staphylococcus aureus	2	2	0	4
Streptococci pneumoniae	0	1	1	2
Staphylococcus viridans	1	0	0	1
Streptococcus pyogens	0	2	0	2
Streptococcus species	4	0	0	4

Table.11: The following table shows percentage of isolated aerobic organisms in three groups.

Isolated organism - Anaerobic culture	Surface of normal oral mucosa (Control group)	Surface of OSCC (OSCC group)	Deeper tissue of OSCC (OSCC group)
Candida species	20.00	3.33	16.67
Coagulase negative staphylococcus species	3.33	0.00	0.00
Enterobacter species	3.33	0.00	0.00
Escherichia coli	0.00	3.33	0.00
Klebsiella pneumonia	20.00	10.00	3.33
Oral commensals	30.00	13.33	3.33
Micrococcus species	3.33	0.00	0.00
Peptostreptococcus species	3.33	3.33	0.00
Pneumococci species	16.67	23.33	6.67
Proteus species	0.00	3.33	3.33
Pseudomonas species	3.33	23.33	6.67
Staphylococcus aureus	6.67	6.67	0.00
Streptococci pneumoniae	0.00	3.33	3.33
Staphylococcus viridans	3.33	0.00	0.00
Streptococcus pyogens	0.00	6.67	0.00
Streptococcus species	13.33	0.00	0.00

Inference from table 10 & 11: Represents frequency and percentage of individual bacteria on the surface of normal oral mucosa, surface of OSCC and deeper tissue of OSCC. In the aerobic group of microorganisms, *Candida* showed higher incidence in the deeper tissues of OSCC. Other species that were more in the deeper tissue were *Pseudomonas, Pneumococci, Proteus, Klebsiella pneumonia, Streptococcus pneumonia and oral commensals.* Microorganism those showed higher incidence on the surface of OSCC were *Pseudomonas, Pneumococci, oral commensals, Klebsiella pneumonia, Staphylococcus aureus, Streptococcus pyogens, Candida, Escherichia coli, Peptostreptococcus, Proteus and Streptococcus pneumonia.* The surface of normal oral mucosa showed predominantly presence of *oral commensals*, *Candida, Klebsiella pneumonia, Pneumococci, Staphylococcus aureus, coagulase negative staphylococcus species, Enterobacter, Peptostreptococcus, Pseudomonas and Streptococcus pyogens.*

Table.12: The following table shows frequency of isolated anaerobic organisms in three groups.

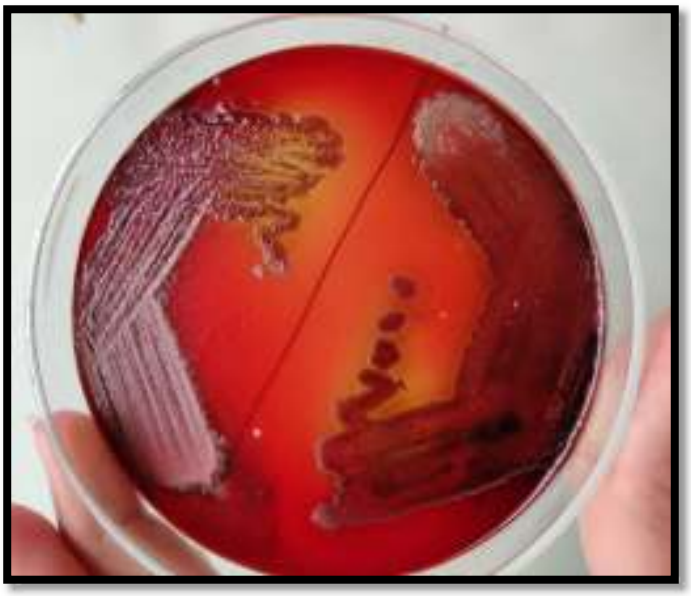
Isolated organism - Anaerobic culture	Surface of normal oral mucosa (Control group)	Surface of OSCC (OSCC group)	Deeper tissue of OSCC (OSCC group)	Total
Bacteroides sp.	0	0	2	2
Fusobacterium sp.	1	1	1	3
Peptostreptococcus anerobius	0	1	0	1
Peptostreptococcus sp.	4	1	1	6
Porphyromonas sp.	1	2	2	5
Prevotella sp.	1	1	2	4

Table.13: The following table shows percentage of isolated anaerobic organisms in three groups.

Isolated organism - Anaerobic culture	Surface of normal oral mucosa (Control group)	Surface of OSCC (OSCC group)	Deeper tissue of OSCC (OSCC group)
Bacteroides sp.	0.00	0.00	6.67
Fusobacterium sp.	3.33	3.33	3.33
Peptostreptococcus anerobius	0.00	3.33	0.00
Peptostreptococcus sp.	13.33	3.33	3.33
Porphyromonas sp.	3.33	6.67	6.66
Prevotella sp.	3.33	3.33	6.67

Inference from table 12 & 13: Represents frequency and percentage of individual microorganism on the surface of normal oral mucosa, surface of OSCC and deeper tissue of OSCC. In anaerobic group, in the deeper tissue of OSCC- *Bacteroides, Porphyromonas, Prevotella and Fusobacterium* species were predominantly found. On the surface of OSCC*, Porphyromonas and Peptostreptococcus anaerobius* were the predominantly seen species, followed by the species *Fusobacterium, Micrococcus and Prevotella*. The surface of normal oral mucosa predominantly showed presence of *Peptostreptococcus species*, followed by *Fusobacterium, Porphyromonas,* and *Prevotella* species.

AEROBIC CULTURE RESULTS



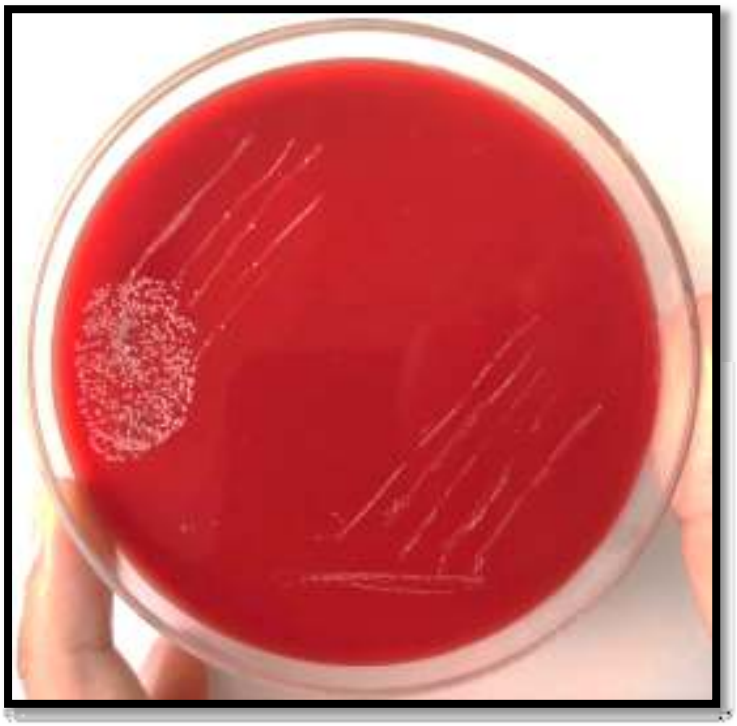
Photograph.9: A- Large irregular flat, alpha hemolytic colonies

B- Large irregular flat, alpha hemolytic colonies

Photograph.10: A.1-Minute circular whitish opaque, non-hemolytic colonies

2-Large irregular flat whitish opaque, non-hemolytic colonies

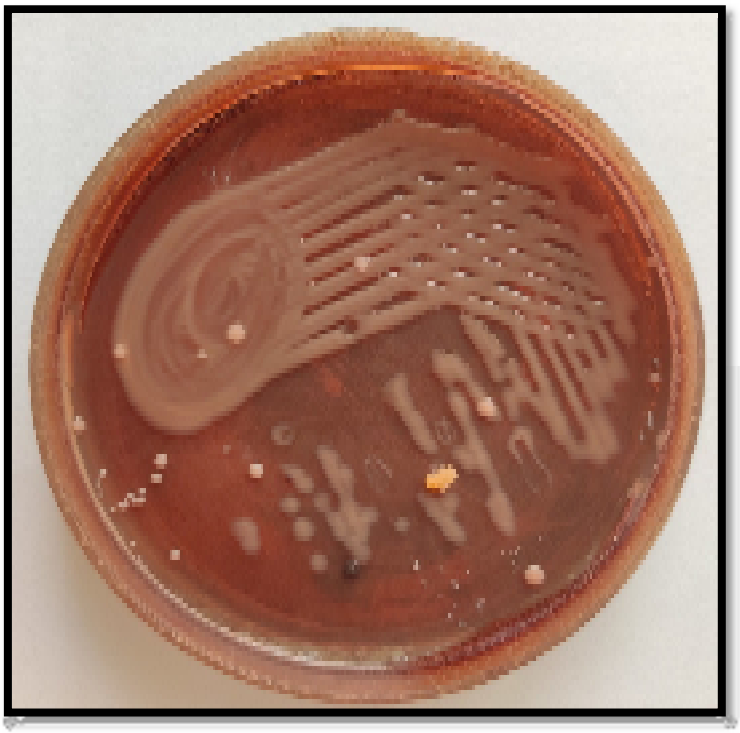
B-Large irregular flat greyish, moist, non-hemolytic colonies



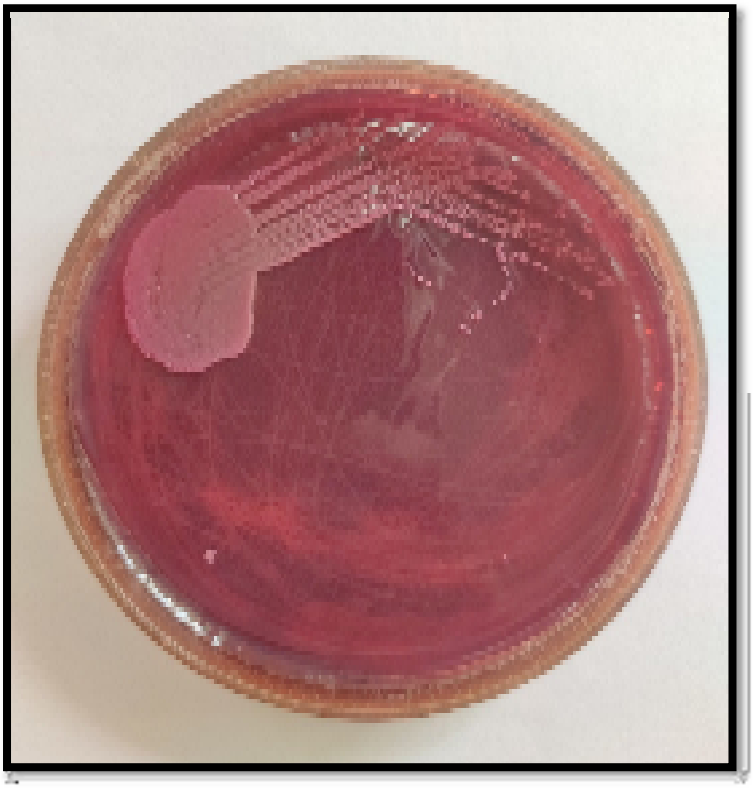
Photograph.11: A-1-Minute circular, whitish opaque non-hemolytic colonies

2-Small circular whitish opaque non –hemolytic colonies

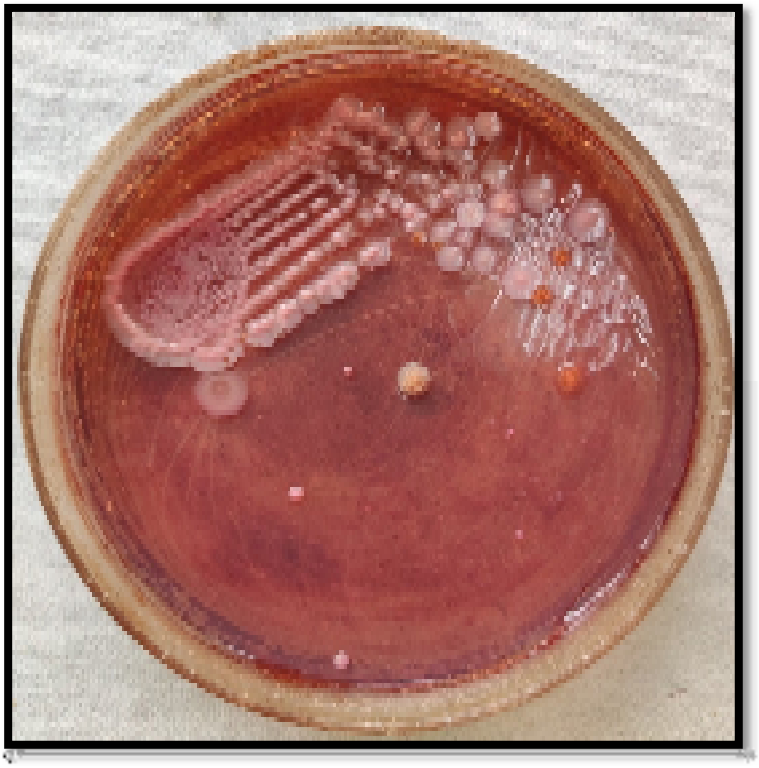
B-Small circular flat whitish opaque non-hemolytic colonies



Photograph.12: Large irregular, mucoid, non-lactose fermenting colonies



Photograph.13: Small circular, mucoid lactose fermenting colonies



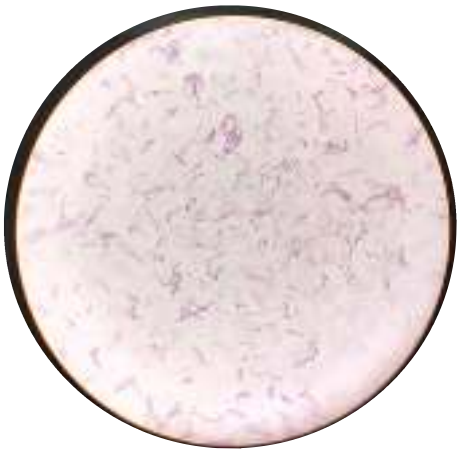
Photograph.14: Large irregular, flat, superimposed lactose fermenting colonies

ANAEROBIC CULTURE RESULTS

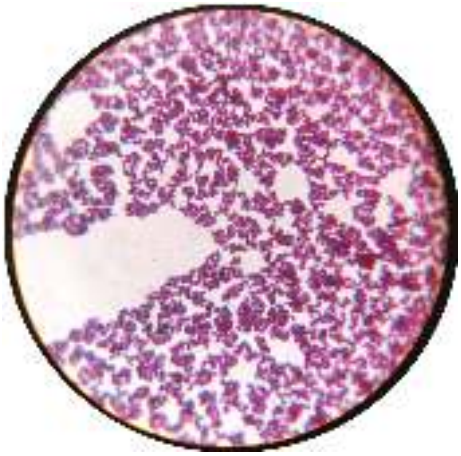
Photograph.15: 1.Small circular blackish opaque non-hemolytic colonies with putrid odour.
2. Small circular whitish opaque Beta-hemolytic colonies

Photograph.16: 1 –Small Circular blackish opaque non-hemolytic colonies with putrid odour.
2 -Small circular whitish opaque Beta-hemolytic colonies

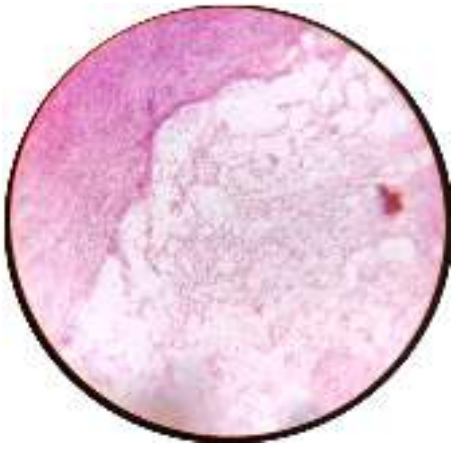
SMEAR PREPARATION AND GRAM'S STAINING



Photograph.17: Gram positive cocci in chains



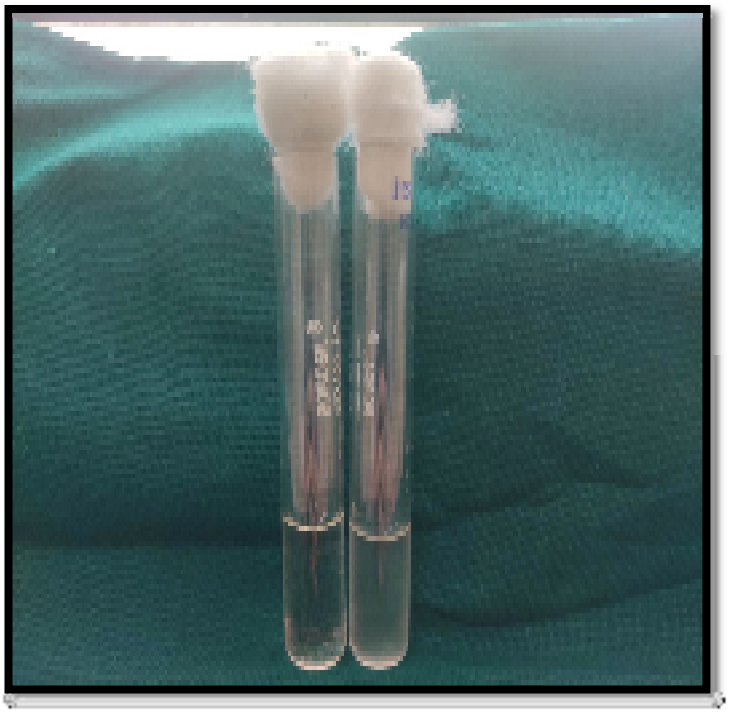
Photograph.18: Budding yeast cells in clusters



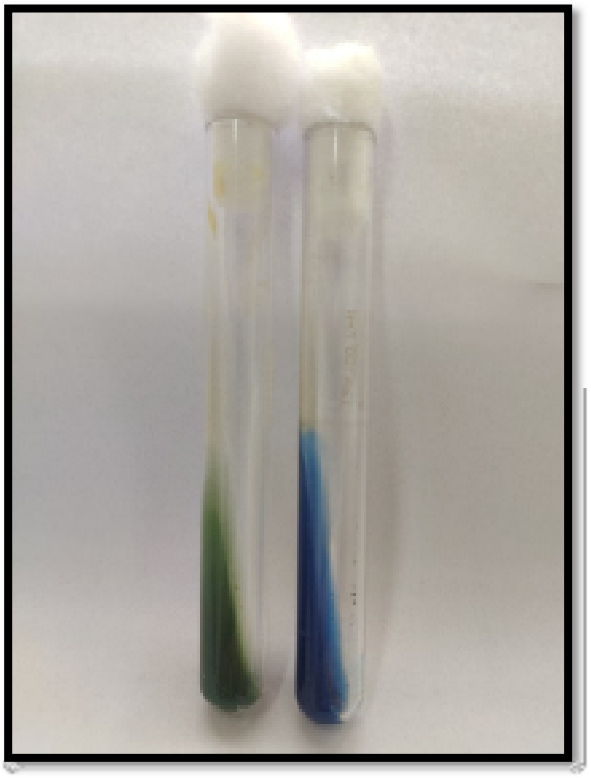
Photograph.19: Gram negative bacilli in clusters

BIOCHEMICAL REACTION FOR BACTERIAL IDENTIFICATION

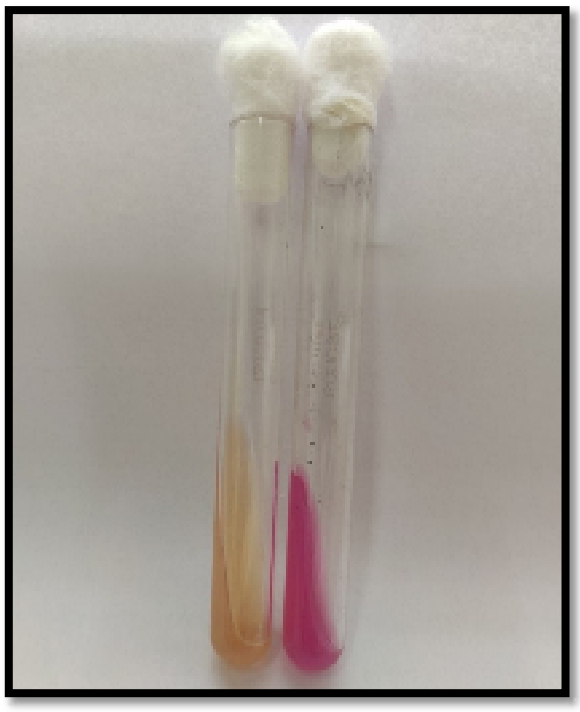
Photograph.20: Various biochemical tests performed for identification of bacterial isolates.



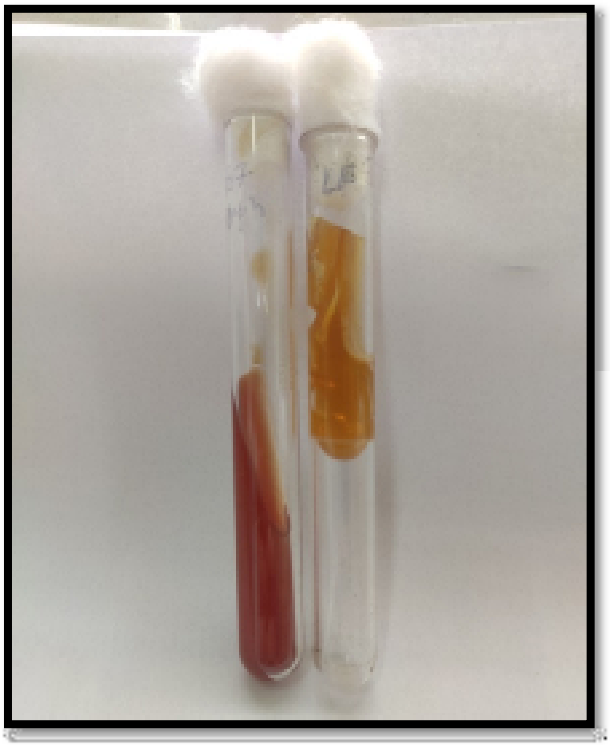
Photograph.21: Peptone water turns turbid when growth of bacterial colonies is seen.



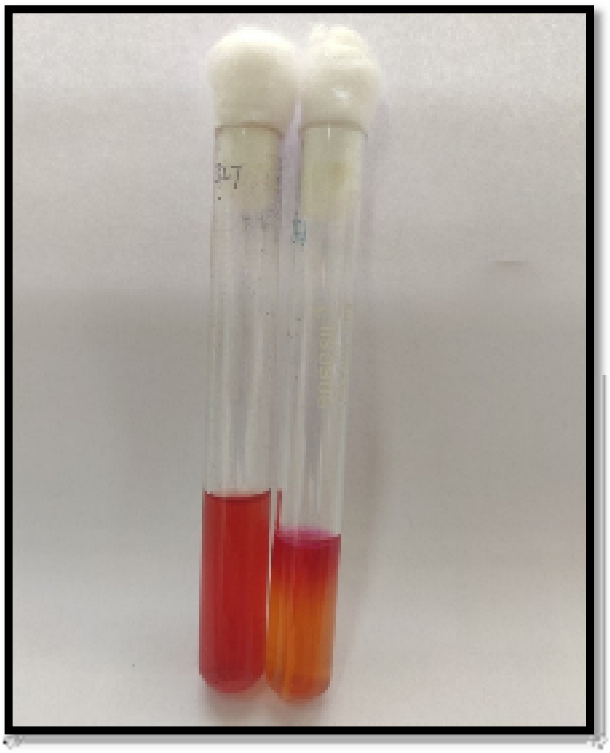
Photograph.22: Citrate test: Performed using Simmonson's citrate medium. When it is positive, color changes from green to blue.



Photograph.23: Urease test: Performed using Christensen's urea medium. When positive, color changes to pink due to presence of phenol red indicator.



Photograph.24: Triple sugar iron test: Inoculated medium shows acid production due to sugar fermentation, color changes to yellow. Here test shows gas produced by bacteria



Photograph.25: Mannitol motility test: It is used to determine motility and mannitol fermentation. When positive, red color clear medium turns slightly yellow opalescent**.**

DISCUSSION:

The oral microbiota is critical in health, and discrepancies among microorganisms and their hosts, results in oral and systemic illnesses. The oral cavity is a harbor for various microorganisms. At different regions within the oral cavity, this varied microbiota exhibits higher qualitative and quantitative variance. In health, however, they attain and maintain a characteristic microflora. Many variables influence the nature and kind of microflora, including pH, oxidation, reduction potential, and nutrient availability. The "bacterial" microbiome resides & is a part of the digestive system and the oral cavity. Bacteria in the gut either live on the epithelium or pass through the lumen, both of which play important but different roles in maintaining host-microbiome homeostasis and have the unique ability to sculpt the systemic immune response from an early age, where constant interactions between the gut microbiome and the mucosa control inflammation and mediate immune tolerance via bacterial translocation. Commensal bacteria help maintain a healthy immune system throughout the body by directing the production of various immune cells that are subsequently needed in the fight against cancer cells. [117]

The present study consisted of 90 samples, in which 30 were from surface of normal oral mucosa (Control group), 30 from surface of OSCC (Sample A) and 30 from the deeper tissue of the same individuals with OSCC (Sample B). The OSCC group consisted of clinically diagnosed and histopathologically confirmed cases. Other demographic details such as age, gender, habit and site from where the sample was taken were noted. The cases in the control group comprised of individuals without any habit history and they were taken from the site of gingivobuccal sulcus.

According to WHO's "*International Agency for Research on Cancer (IARC)*", in the year 2020, the incidence of lip and oral cavity carcinoma cases among males in the South-Eastern Asia region were 11,297 and those among females was 7084 i.e., it showed more incidence of disease in males than in females. In the OSCC group of the current study, maximum number of individuals were males as seen in table 2, which showed frequency and percentage of gender distribution among OSCC individuals, the males were 4 times more than females in our study. This is in accordance with the report of incidence & mortality for oral cancer from IARC. [118]

Similarly, in another study by **Singh et al., 2015;** [119]who retrospectively evaluated OSCC cases in India, showed higher prevalence in males (75.9%) than in females (24.1%) which is similar to that seen in our study. The reason for this, can probably be due to the increasing intake of tobacco among men. However, smokeless tobacco use is also an important risk factor in females. [119]

In the present study, as shown in table 1, the age range 51-60 years had the highest number of OSCC cases, i.e., more frequent in the 6th decade as seen in table 1. This was in correlation with the statement by **Smitha et al., 2017**, [120] who retrospectively examined the clinicopathological features of OSCC in South India. However, off late there has been an increasing trend in OSCC in younger individuals

Babu et al., 2021, [121] studied the epidemiological trends in OSCC, in the Southern part of India, which showed a male-to-female proportion of 0.7: 0.3, inferring to male predominance, with highest number of cases occurring in the 4th and 6th decade of life and the most commonly affected site being buccal mucosa i.e., in 40% of the cases. All the findings were in accordance with the current study as shown in table 1, 2 and

3. Many other studies conducted in various parts of India, confirmed these findings. [122-126]

In the OSCC group, 73.33% (table 4) of the subjects had a history of consuming tobacco in any form i.e. smoking or smokeless tobacco products, this may be due to the socioeconomic conditions or due to the readily availability of various tobacco products in this region. Whereas, 26.77 % of the subjects did not possess any tobacco consumption habits. Occurence of OSCC in such individuals may be attributed to various other etiological factors as mentioned in the review of literature.

Similar age and gender distribution was seen in the control group, with maximum number of cases seen in the 5^{th} and 6^{th} decade of life as seen in table 5. A higher number of individuals being males as seen in table 6. All the cases in the control group were taken from the gingivobuccal sulcus area and the individuals with no habit history were chosen as controls, to avoid habit as a confounding factor.

A mean age of 51.10 & deviation from the standard of 10.88 in OSCC group, and mean age of 45.67 with standard deviation of 11.56 was seen in the control group as depicted in Table 7 & 8 that shows comparison of frequency, percentage and mean age distribution in OSCC and control group using Chi-square test & t-test in the present study, this infers that there is a difference in the mean age among both the groups, similar to that seen in another study. [127]

Table 9 compares the gender distribution in OSCC and control group of our study, and it shows higher number of cases are seen among males in both the groups. This was in accordance to a study done by **Yang et. al, 2018;** [127] who evaluated the

oral microbiota in OSCC and healthy controls further profiled it using 16S rRNA technique.

The present study evaluated the microflora present on the surface of normal oral mucosa, it predominantly showed presence of *Candida sp.,* along with aerobic microorganisms such as *Klebsiella pneumonia, Pneumococci, Staphylococcus aureus, coagulase negative Staphylococcus species, Enterobacter, Micrococcus, Peptostreptococcus, Pseudomonas and Streptococcus pyogens.* Anaerobic microorganisms predominantly consisting of *Fusobacterium, Porphyromonas* and *Prevotella* species were found. (Table 10,11,12 & 13)

Aas et al., 2005;[128] utilized culture-independent molecular techniques to discover the microbial community in a healthy oral cavity of humans, which also included uncultured bacteria. The survey discovered 141 dominating species, with over 60% of them having never been grown. A total of thirteen new lineages have been discovered. Species characteristically linked with oral diseases were not discovered. As opposed to the findings in our study which showed presence of genera *Fusobacterium, Porphyromonas* and *Prevotella* .

As shown in tables 10, 11, 12 & 13, the surface of OSCC, showed presence of *Pseudomonas, Pneumococci, Klebsiella pneumonia, Staphylococcus aureus, Streptococcus pyogens, Escherichia coli, Micrococcus, Peptostreptococcus, Proteus and Streptococcus pneumonia* among the aerobic microorganisms. Fungi such as *Candida sp.,* was also seen. *Porphyromonas and Peptostreptococcus anerobius* were the predominantly seen species, followed by the species *Fusobacterium, and Prevotella,* among the anaerobic group of microorganisms.

On comparison, the surface of normal oral mucosa differed from that of surface of OSCC, as pathogenic microorganisms such as *Pneumococci* and *Pseudomonas* species were seen in higher numbers on surface of OSCC, whereas the surface of normal oral mucosa showed presence of commensal microorganisms such as *Candida* species. However, the surface also showed some pathogenic bacteria such as *Klebshiella pneumonia, Streptococcus* species which may be due to contamination of the sample.

Nagy et al., 1998,[66] published the first study on the microbiological components of biofilm communities found on the surface of OSCC. The median number of anaerobic colony forming units at tumour locations was substantially greater than the healthy oral mucosa, the same was true for aerobic microorganism at tumour locations relative to the controls. *Veillonella, Fusobacterium, Prevotella, Porphyromonas, Actinomyces,* and *Clostridium* were among the species isolated in greater quantities in tumour locations among the anaerobes Aerobic microorganisms such *Haemophilus, Enterobacteriaceae* and *Streptococcus spp., Candida albicans* was also found at the tumor locations. Some microorganisms such as *Candida sp., Streptococcus sp., Fusobacterium* and *Prevotella* were similar to those found in our study.

Bolz et al., 2013,[129] identified the microbial profiles on the surfaces of OSCC, compared it with individuals with higher risk of developing OSCC such as heavy smokers, alcohol consumers who haven't developed OSCC yet. A third group of healthy controls were also evaluated. In the control groups, the ratio of aerobes to anaerobes was roughly 2:1, whereas in the group at high risk, the proportion was nearly balanced. In the group of oral squamous cell carcinoma, an inverse ratio was

discovered. They investigated at the rates of bacterial resistance in *Staphylococcus, E. coli, Enterococcus, Haemophilus parainfluenzae*, and *Prevotella*. Healthy controls had the lowest resistance, which rose in high-risk persons, and was highest in OSCC. Microorganisms such as *Staphyloccocus* and *Prevotella* species were also seen in our study. Another original study, investigated the OSCC subgroup and came up with similar conclusions. [154] Using 16S rRNA sequencing, they evaluated the association of oral bacteria in OSCC tissue and compared it to nearby non-tumor tissue 5cm away from the lesion in the same patient.

Fungi such as *Candida sp.* showed higher incidence in the deeper tissues of OSCC. Other aerobic species that were more in the deeper tissue were *Pseudomonas, Pneumococci, Proteus, Klebsiella pneumonia* and *Streptococcus pneumonia.* In anaerobic group, *Bacteroides, Porphyromonas, Prevotella* and *Fusobacterium* species were predominantly found in the current study.

Till date, only **Hooper et al**., **2006,** [70]identified the presence of viable bacteria within the OSCC tissue. It was a preliminary study conducted, which used standard microbiological method for identification of bacteria. Then those isolates were sequenced using 16S rRNA molecular technique. *Yeasts, Actinomycetes, Bifidobacteria, Lacobacilli, Streptococci,* and *Veillonellae* were fermentative and acid-resistant type of bacteria that were discovered.

Saccharolytic microorganisms are the ones that utilize or ferment sugars for their metabolism, few examples are *Bifidobacterium, Lactobacilli, Clostridium* and *Eubacterium.* Asacchrolytic microorganisms are the ones that do not require carbohydrates or sugars for their metabolism. Anaerobic gram positive species,

Bacteroides and perio-pathogens such as *Porphyromonas gingivalis* are one such examples for asacchrolytic bacteria.

In contrast to the findings of **Hooper et al**, the present studied showed presence of asacchrolytic bacteria such as *Fusobacterium, Porphyromonas, Bacteriodes* and *Prevotella.*

On comparison, the deeper tissue of OSCC showed presence of viable species of *Candida*, which has not been demonstrated in any other studies, strongly points towards the possible role of *Candida* in oral cancer development and progression. Earlier studies done by **Sankari et al., 2020,** [130] showed increased Candidal carriage in saliva of OSCC individuals, however none have evaluated in deeper tissue of OSCC.

Presence of periodontitis related bacteria such as *Fusobacterium, Porphyromonas, Bacteriodes* and *Prevotella,* were also seen in deeper tissues of OSCC when compared to the surface of OSCC showing *Pneumococci* and *Pseudomonas* species in our study. The role of *Fusobacterium and Porphyromonas* in the progression of OSCC is described earlier in the studies done by **Chen et al., 2021**,[87] & **Neuzillet et al., 2021.** [113] (discussed on review of literature page 15-17) However, the role of other microorganisms in OSCC is yet to be explored.

Zhao et al., 2017, [131] used Next Generation Sequencing (NGS) to explore the oral microbial dysbiosis in OSCC and healthy individuals, with the goal of better understanding the bacterial community composition and associated functional genes. It revealed that OSCC patients have much more bacterial diversity than typical healthy adults. *Fusobacterium, Dialister, Peptostreptococcus, Filifactor,*

Peptococcus, Catonella, and *Parvimonas* were identified to be associated with periodontal infections. Other studies also implicated the bacteria *Fusobacterium nucelatum, Pseudomonas aeroginosa*, and *Fustobacterium periodonticum* to the development of OSCC. [132]

Various attempts are being made using PCR technology, DNA hybridization and other culture-independent methods to describe the oral microflora, but experiments have found very limited bacterial changes. [133-135]

The conclusion of the present study is that we were successfully able to isolate certain fungi, asacchrolytic microorganisms and anaerobic bacteria such as *Fusobacterium* and *Porphyromonas* in deeper tissue of OSCC. This warrants further research into the role of these microorganisms.

SUMMARY AND CONCLUSION:

- Aim of the present research was to evaluate bacterial microflora on the surface of normal oral mucosa (NOM), OSCC and in deeper tissues of OSCC utilizing a standard microbiological culture method.

- A total of 90 samples were cultured. 30 each from surface of NOM, OSCC and deeper tissues of OSCC.

- The OSCC group included a higher proportion of men, with a higher number of cases in the 51-60 year age range. Buccal mucosa being the most commonly affected site in the present study. Most of the individuals with OSCC had a history of tobacco consumption of any form.

- The surface of normal mucosa showed presence of *Candida sp.,* along with aerobic microorganisms such as *Klebsiella pneumonia, Pneumococci, Staphylococcus aureus, coagulase negative staphylococcus species, Enterobacter, Micrococcus Peptostreptococcus, Pseudomonas and Streptococcus pyogens.* Anaerobic microorganisms such as *Peptostreptococcus species*, *Fusobacterium, Porphyromonas,* and *Prevotella* species were also found.

- The surface of oral squamous cell carcinoma showed presence of aerobic microorganisms such as *Pseudomonas, Pneumococci, Klebsiella pneumonia, Staphylococcus aureus, Streptococcus pyogens, Escherichia coli, Micrococcus, Peptostreptococcus, Proteus and Streptococcus pneumonia.*. Fungi such as *Candida sp.,* was also seen. Anaerobic microorganisms such as *Porphyromonas*

and Peptostreptococcus anerobius, Fusobacterium, and Prevotella were identified.

- Deeper tissue of OSCC showed presence of *Candida sp, Pseudomonas, Pneumococci, Proteus, Klebsiella pneumonia and Streptococcus pneumonia.(aerobes). Bacteroides, Porphyromonas, Prevotella and Fusobacterium* species (anaerobes) were also identified.

- The study concludes that a group of asacchrolytic, periodontitis related microorganisms were found along with increased number of *Candida* species in deeper tissues of OSCC.

- However, there is still a need to further investigate the role of these microbes in the development, progression and prognosis of the disease.

LIMITATIONS:

- The limitation of the current study is reliance on culture-dependent method for identification of bacterial microflora. The use of newer molecular technologies such as 16S rRNA sequencing and Next generation sequencing could not be employed due to the higher cost and time restraint.

FUTURE SCOPE:

1. Use of 16S rRNA sequencing in identification of species within deeper tissue of OSCC.
2. Quantitative analysis of microbial load on surface and deeper tissues of OSCC.
3. Explore the potential use of microorganisms as a technique for early detection diagnostics of OSCC.
4. The correlation of prognostic factors such as recurrence, metastasis and overall survival with presence of microorganisms.

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ANNEXURE-I

CASE HISTORY PERFORMA

Case No: **OPD No:** **Date:**

Name:

Age: **Sex:** **Occupation:**

Marital status:

Address:

Chief Complaint:

History of presenting illness:

Past medical & dental history:

Personal habits:

General Physical Examination:

Extraoral Examination:

Intraoral examination:

Hard tissue examination:

Soft tissue examination:

Provisional diagnosis:

Investigations:

Final diagnosis:

CLINICAL PARAMETERS:

New primary/Recurrence/Previous Treatment:

Clinical TNM stage:

Histological type:

Differentiation- Well:

Moderate:

Poor:

Lymph node metastasis

ANNEXURE II - BISTATISTICS CLEARANCE CERTIFICATE

KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
Re-Accredited 'A' grade by NAAC (2nd Cycle) & Placed in Category 'A' by MHRD (GoI)
0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail: principal@kledental-bgm.edu.in

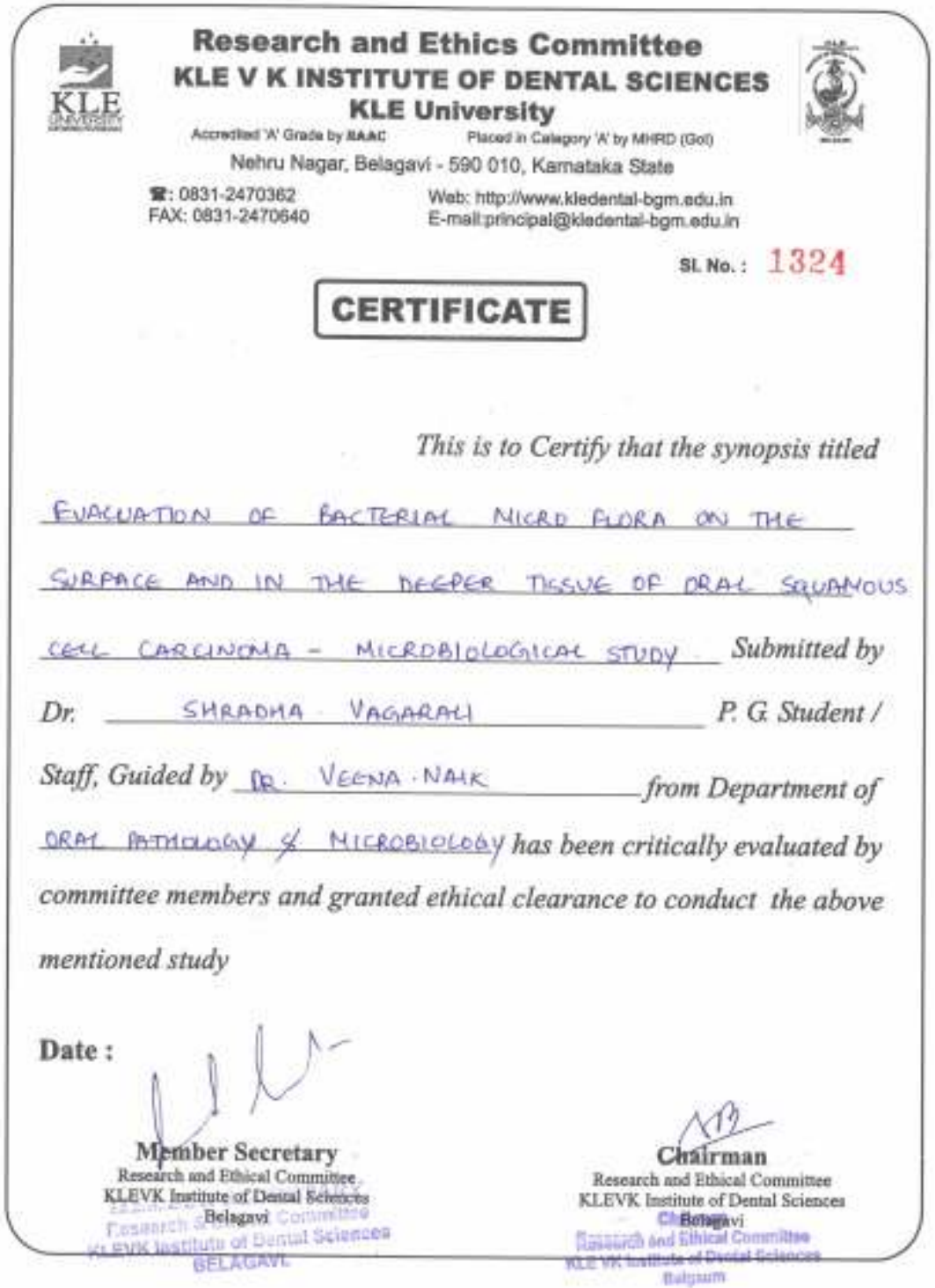
Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of **Dr. Shradha Vagarali, Post Graduate Student**, under the guidance of **Dr. Veena V Naik, M.D.S. Professor, Department of Oral and Maxillofacial Pathology and Oral Microbiology** entitled **"Evaluation of Bacterial Microflora from Surface of Normal Oral Mucosa, Oral Squamous Cell Carcinoma and Deeper Tissues Of Oral Squamous Cell Carcinoma- A Microbiological Study"** has been done under my guidance and considered satisfactory.

Place: Belagavi
Date: 14/12/2021

Name & Signature of Biostatistician
(Dr. S. B. Javali)

ANNEXURE III - ETHICAL CLEARANCE CERTIFICATE



Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
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Sl. No.: 1324

CERTIFICATE

This is to Certify that the synopsis titled

EVALUATION OF BACTERIAL MICRO FLORA ON THE SURFACE AND IN THE DEEPER TISSUE OF ORAL SQUAMOUS CELL CARCINOMA - MICROBIOLOGICAL STUDY *Submitted by*

Dr. SHRADHA VAGARALI *P. G. Student / Staff, Guided by* DR. VEENA NAIK *from Department of* ORAL PATHOLOGY & MICROBIOLOGY *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date :

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE-IV

INFORMED CONSENT FORM

Principal Investigator : Dr. SHRADHA VAGARALI

I _____, aged _____ years have been informed about my involvement in the study.

I agree to give my personal details like Name, Age, Gender, Residential Address, past and present dental history and any other details if required for the study to the best of my knowledge.

I will co-operate with the dentist.

I will follow the instructions given by the dentist during study.

I will visit the dentist as and when required for the study, at the given time and date.

I permit the dentist to utilize the information given and results obtained from this study for presentation and publication without disclosing my identity.

I have understood the nature of the study and permit the dentist to carry out the required surgical procedure.

If for any reason I am unable to participate in the study, for reasons unknown, I can withdraw from the study at any given point of time.

I have been informed that an **oral swab** will be taken from my oral cavity and after surgery **a piece of tumor tissue** will be taken for **bacterial culture**, after understanding the procedure, I permit the dentist to perform the same.

If by chance any complications arise during the above said procedure, I permit the dentist to take necessary actions to prevent the same.

In my full consciousness and presence of mind, after understanding all the procedures and related complications if any, in my vernacular language, I am willing and give my consent to participate in this study.

Date:
Name of the Patient:
Signature:
Address & Ph. No

INFORMED CONSENT FORM (IN KANNADA)

ಮಾಹಿತಿ ಕಾನ್ಸೆಂಟ್ ಫಾರ್ಮ್

ಪ್ರಧಾನ ತನಿಖಾಧಿಕಾರಿ : ಡಾ. ಶ್ರದ್ಧಾ ವಾಗರ್ಲಿ

ನಾನು _____, ವಯಸ್ಸಿನ _____ ವರ್ಷಗಳ ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ತೊಡಗಿರುವ ಬಗ್ಗೆ ತಿಳಿಸಲಾಗಿದೆ.

ನನ್ನ ಜ್ಞಾನದ ಅತ್ಯುತ್ತಮ ಅಧ್ಯಯನಕ್ಕೆ ಅಗತ್ಯವಿದ್ದರೆ ನನ್ನ ವೈಯಕ್ತಿಕ ವಿವರಗಳನ್ನು ಹೆಸರು, ವಯಸ್ಸು, ಲಿಂಗ, ವಸತಿ ವಿಳಾಸ, ಹಿಂದಿನ ಮತ್ತು ಪ್ರಸ್ತುತ ದಂತ ಇತಿಹಾಸ ಮತ್ತು ಇತರ ವಿವರಗಳನ್ನು ನೀಡಲು ಒಪ್ಪುತ್ತೇನೆ.

ನಾನು ದಂತವೈದ್ಯರೊಂದಿಗೆ ಸಹಕರಿಸುತ್ತೇನೆ.

ಅಧ್ಯಯನದಲ್ಲಿ ದಂತವೈದ್ಯ ನೀಡಿದ ಸೂಚನೆಗಳನ್ನು ನಾನು ಅನುಸರಿಸುತ್ತೇನೆ.

ನಾನು ನಿರ್ದಿಷ್ಟ ಸಮಯದಲ್ಲಿ ಮತ್ತು ದಿನಾಂಕದಂದು ದಂತವೈದ್ಯರಿಗೆ ಮತ್ತು ಅಧ್ಯಯನಕ್ಕೆ ಅಗತ್ಯವಿದ್ದಾಗ ನಾನು ಭೇಟಿ ನೀಡುತ್ತೇನೆ.

ನನ್ನ ಗುರುತನ್ನು ಬಹಿರಂಗಪಡಿಸದೆ ಪ್ರಸ್ತುತಿ ಮತ್ತು ಪ್ರಕಟಣೆಗಾಗಿ ಈ ಅಧ್ಯಯನದಿಂದ ಪಡೆದ ಮಾಹಿತಿ ಮತ್ತು ಫಲಿತಾಂಶಗಳನ್ನು ಬಳಸಿಕೊಳ್ಳಲು ನಾನು ದಂತವೈದ್ಯರಿಗೆ ಅನುಮತಿ ನೀಡುತ್ತೇನೆ

ನಾನು ಅಧ್ಯಯನದ ಸ್ವಭಾವವನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ ಮತ್ತು ಅಗತ್ಯ ಶಸ್ತ್ರಚಿಕಿತ್ಸಾ ಕಾರ್ಯವಿಧಾನವನ್ನು ಕೈಗೊಳ್ಳಲು ದಂತವೈದ್ಯರನ್ನು ಅನುಮತಿಸುತ್ತೇನೆ.

ಯಾವುದೇ ಕಾರಣಕ್ಕಾಗಿ ನಾನು ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಸಾಧ್ಯವಾಗದಿದ್ದರೆ, ಕಾರಣಗಳು ಅಜ್ಞಾತವಾಗಿದ್ದಲ್ಲಿ, ಯಾವುದೇ ಸಮಯದಲ್ಲಾದರೂ ಅಧ್ಯಯನದಿಂದ ನಾನು ಹಿಂತೆಗೆದುಕೊಳ್ಳಬಹುದು.

ಅನ್ನು ಬಳಸಿಕೊಂಡು ಮೂಳೆಯ ದೋಷದ ಉದ್ದೇಶಕ್ಕಾಗಿ ನನ್ನ ಮೇಲೆ ನಡೆಸಿದ ಶಸ್ತ್ರಚಿಕಿತ್ಸಾ ಪ್ರಕ್ರಿಯೆಯು ತುಲನಾತ್ಮಕವಾಗಿ ಹೊಸ ವಸ್ತುವಾಗಿದೆ ಮತ್ತು ಕಾರ್ಯವಿಧಾನವನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡ ನಂತರ ನಾನು ದಂತವೈದ್ಯರನ್ನು ಅದೇ ರೀತಿ ಮಾಡಲು ಅನುಮತಿಸುತ್ತೇನೆ ಎಂದು ನನಗೆ ತಿಳಿಸಲಾಗಿದೆ.

ಮೇಲೆ ತಿಳಿಸಿದ ಪ್ರಕ್ರಿಯೆಯಲ್ಲಿ ಯಾವುದೇ ತೊಂದರೆಗಳು ಉಂಟಾದರೆ, ದಂತವೈದ್ಯರು ಅದನ್ನು ತಡೆಯಲು ಅಗತ್ಯ ಕ್ರಮಗಳನ್ನು ತೆಗೆದುಕೊಳ್ಳುವಂತೆ ನಾನು ಅನುಮತಿಸುತ್ತೇನೆ.

ನನ್ನ ಸಂಪೂರ್ಣ ಪ್ರಜ್ಞೆ ಮತ್ತು ಮನಸ್ಸಿನ ಉಪಸ್ಥಿತಿಯಲ್ಲಿ, ಎಲ್ಲ ವಿಧಾನಗಳು ಮತ್ತು ಸಂಬಂಧಿತ ಸಮಸ್ಯೆಗಳನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡ ನಂತರ, ನನ್ನ ದೇಶೀಯ ಭಾಷೆಯಲ್ಲಿ, ನಾನು ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನನ್ನ ಒಪ್ಪಿಗೆಯನ್ನು ನೀಡುತ್ತೇನೆ ಮತ್ತು ನನ್ನ ಒಪ್ಪಿಗೆಯನ್ನು ನೀಡುತ್ತೇನೆ.

ಹೆಸರು:
ಸಹಿ:
ವಿಳಾಸ:

INFORMED CONSENT FORM (IN MARATHI)

मुख्य अन्वेषक: डॉ.श्रध्दा वगरली

- मी_____,व _____ वर्ष ल माझ्या वरील संशोधनात सहभागी होण्याची कल्पना दिलेली आहे.
- मी माझे वैयक्तिक माहिती, जसे नाव,व,लिंग,घरचा पत्ता, पूर्व आणि सध्याचा चालू असलेल दंत इतिहास आणि संशोधनासाठी आवश्यक असलेली सर्व माहिती देऊ इच्चीतो/ते.
- मी दंतचिकित्सकांना त्यांच्या अभ्यासात सहकार्य करीन.
- मी दंतचिकित्सकांनी दिलेल्या सर्व सूचना पाळीन.
- मी दंतचिकित्सकांनी संशोधनाकरित जेव्हा बोलावलं तेव्हा दिलेल्या दिवशी आणि वेळेत येईन.
- मी दंतचिकित्सकांना मी दिलेली माहिती आणि संशोधनात मिळालेले परिणाम माझी ओळख उघड न करता प्रसारण करण्याची परवानगी देतो/ते .
- मल दंत्विक्त्सकांना अभ्यास समजल आहे आणि त्यासाठी आवश्यक असलेली शास्त्रक्रिया अमलात आणणे आणि त्यासाठी दंतचिकित्सकांना मी परवानगी आहे.
- कोणत्याही करणस्पती मी संशोधनात सहभागी होऊ शकले नाही (अज्ञात कारणांमुळे), तर मी कोणत्याही वेळी संशोधन सोडू शकतो/ते.
- मल सूचित केले गेले आहे की तोंडी जमीन आणि ट्यूमरचा तुकडा घेतले जाईल आणि प्रक्रिया समजून घेतल्यानंतर मी दंतचिकित्सक ांना तसे करण्याची परवानगी देतो.
- वरील नमुद केलेल्या प्रक्रिये दरम्यान कोणतीही समस्या उद्भवली तर मी दंतचिकित्सकांना झालेली समस्या टाळण्यास आवश्यक कार्यवाही करण्यास परवानगी देतो.
- मी पुर्ण शुद्दीत आणि मनाच्या उपस्थिती, माझ्या मातृभाषेत सर्व प्रक्रिया आणि संबंधित समस्या समजून घेतल्यानंतर, मी या अभ्यासात भाग घेण्यासाठी माझी इच्छा व्यक्त करतो/ते

तारीख :- स्वाक्षरी :-

निवास/पत्ता- पेशंटचे नाव:-

ANNEXURE-V
Patient information sheet

"**Evaluation of bacterial microflora from the surface of normal oral mucosa, oral squamous cell carcinoma and deeper tissues of oral squamous cell carcinoma- A microbiological study"**

Title of the study Evaluation of bacterial microflora from the surface of normal oral mucosa, oral squamous cell carcinoma and deeper tissues of oral squamous cell carcinoma- A microbiological study

Aim of the study: Evaluation of the bacterial microflora on the surface of normal oral mucosa, oral squamous cell carcinoma and in deeper tissue of oral squamous cell carcinoma using standard microbiological culture approach.

Description of the study:

The patients that can be included in this study and are interested to participate will be informed about the study and the protocol that will be followed. We are aiming to include 126 patients, 42 patients of normal oral mucosa of matching age, sex and socio-economic status as that of patients with oral squamous cell carcinoma and 42 patients with clinically diagnosed and histopathologically confirmed cases of oral squamous cell carcinoma. A oral swab will be taken from all the patients after rinsing the oral cavity with normal saline and a tissue specimen 1cm deeper from the site of lesion from patients having oral squamous cell carcinoma will be taken for bacterial culture. The type of bacteria present will be noted.

Participation and Termination:

Your participation in the study is voluntary. You can refuse to participate or ask your study doctor to end your participation before the final closure of the study at any time. Refusal to participate or early termination will not in any way, influence your relationship with and or your treatment by the doctor. If you agree to participate, you will be asked to sign the informed consent form. You have the right to ask questions about this study at any time.

Cost of participation

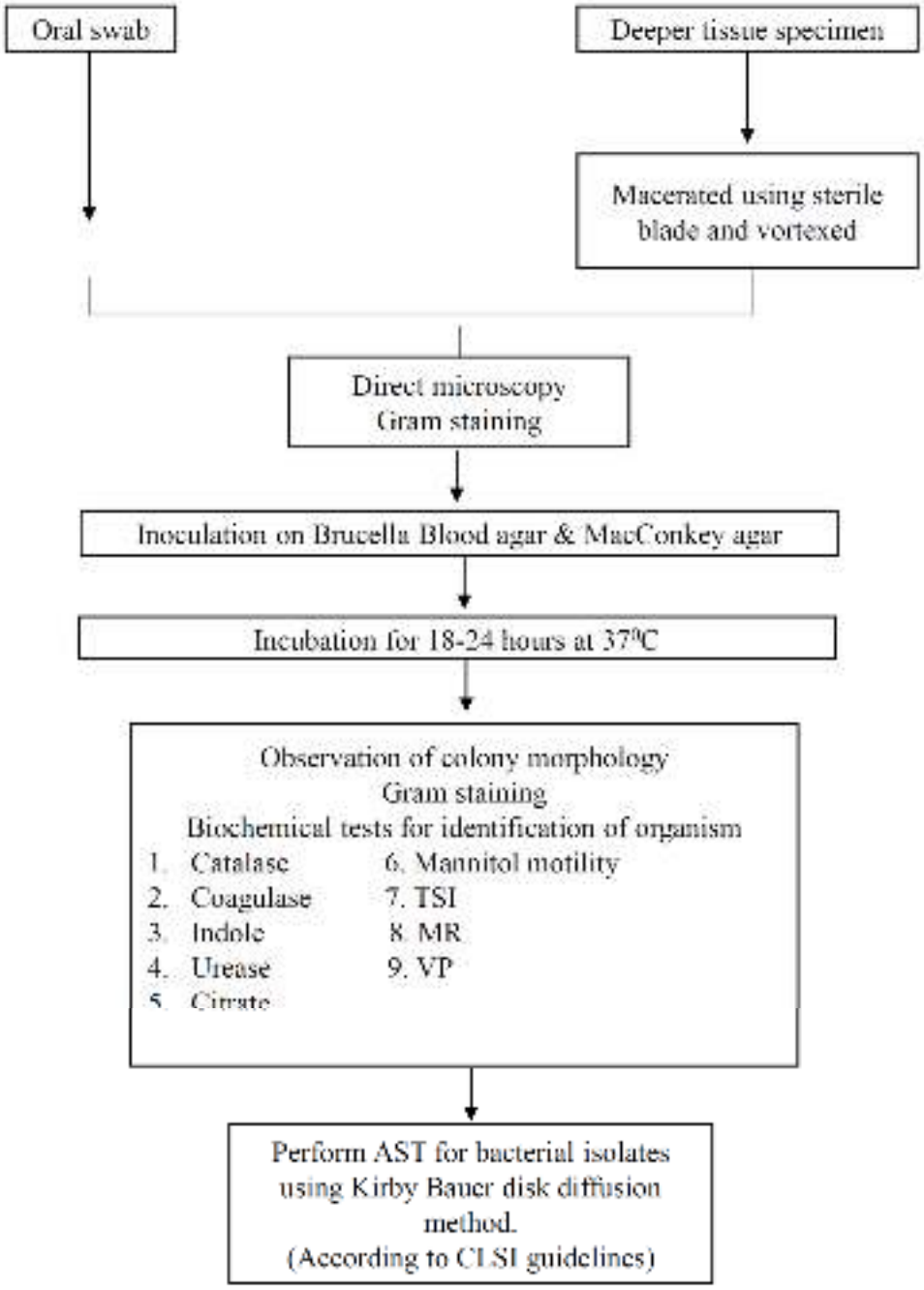
Participating in this trial will not result in any additional cost to you.

Confidentiality

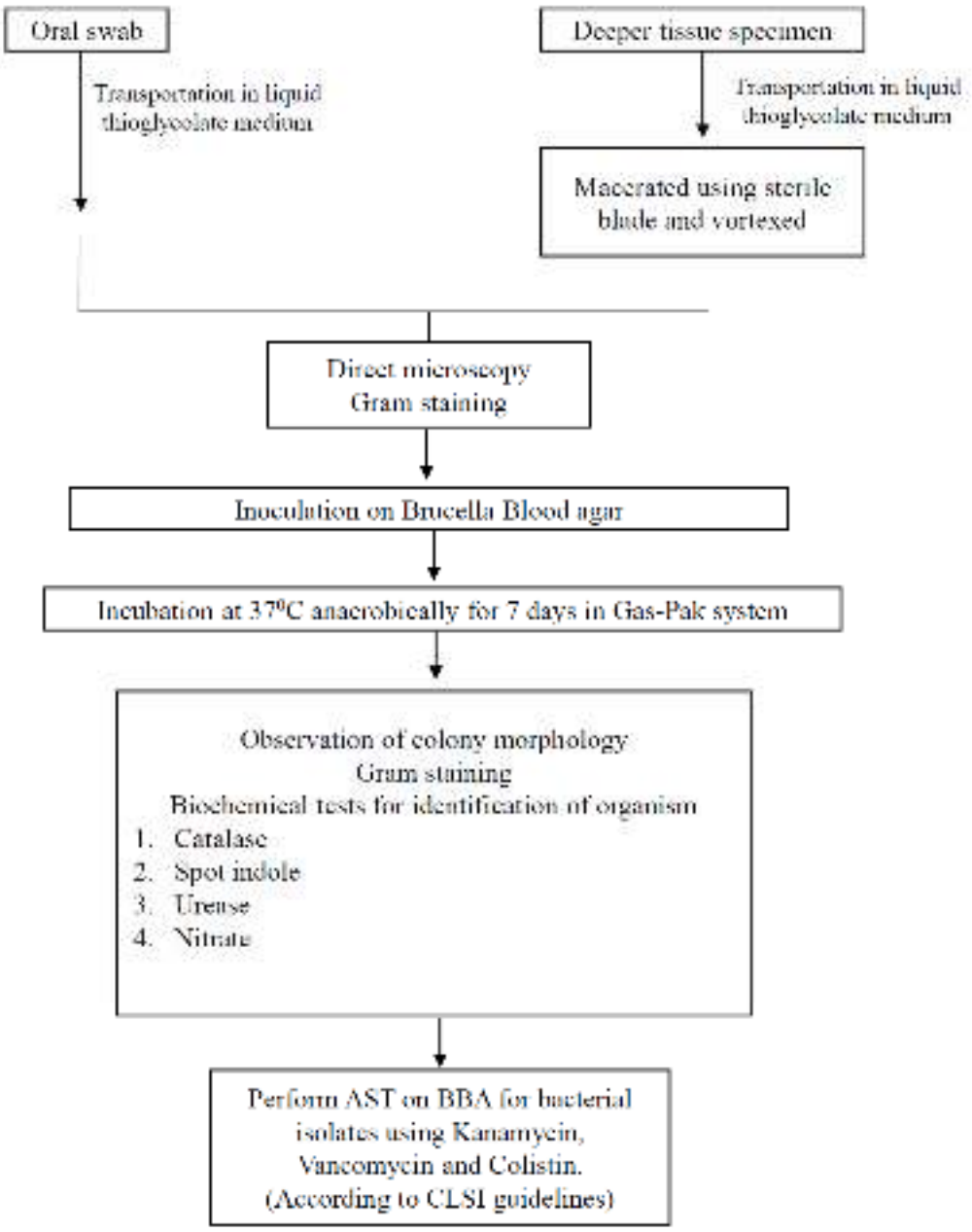
In accordance with Belgian Law concerning private life protection (1992) and patient's rights, information collected from your participation in this study is protected. If you agree to participate in this study, your personal data and clinical information will be collected and coded. When the results of this study will be published, your identity will remain confidential.

ANNEXURE-VI

OVERVIEW OF SAMPLE PROCESSING-AEROBICALLY



OVERVIEW OF SAMPLE PROCESSING-ANAEROBICALLY



ANNEXURE-VII

Preparation of various culture media and media for biochemical reactions

Preparation Of Fluid Thioglycolate Medium

1. Suspend 29.75 grams of Fluid thioglycolate medium powder(Manufacturer-HiMedia) in 1000 ml purified /distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Sterilize by autoclaving at 15lbs pressure (121^0C) for 15 minutes.
4. Cool at 25^0C and store in cool dark place preferably below 25^0C

Preparation Of MacConkey Agar

1. Suspend 51.53 grams of MacConkey agar powder(Manufacturer-HiMedia) in 1000ml distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Sterilize by autoclaving at 15lbs pressure (121^0C) FOR 15 minutes.
4. Mix well before pouring.

Preparation Of Brucella Agar Base W/ Hemin and Vitamin K1

1. Suspend 43.12 grams of Brucella agar base powder in 1000ml purified/distilled water
2. Heat to boiling to recommended for Brucella to dissolve in the medium completely.
3. Sterilize by autoclaving at 15lbs pressure spices for isolation and subculture of (121^0C) for 15 minutes.

4. Cool to 45-50^{0}C and aseptically add 5% v/v sterile anaerobes with addition of blood defibrinated sheep blood. Mix well before pouring inti sterile petri plates.

Preparation Of Urea Agar Base(Christensen)(Autoclavable)

1. Suspend 24.01 grams of Urea Agar (Manufacturer-HiMedia) in 950ml of distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Sterilize by autoclaving at 115 ^{0}C for 20 minutes .Cool at 50^{0}C and aseptically add 50ml of sterile 40% Urea Solution (FD048) and mix well.
4. Dispense into sterile tubes and allow to set in the standing position.
5. DO NOT OVERHEAT OR REHEAT the medium as urea decomposes very easily. Corresponds to 10lbs pressure

Preparation Of Mannitol Motility Test Medium

1. Suspend 26.04 grams of Mannitol Motility agar powder(Manufacturer-HiMedia) in 1000ml of distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (1210C) for 15 minutes. Allow the tubes to cool in an upright position

Preparation Of Triple Sugar Iron Agar

1. Suspend 64.52 grams Triple sugar iron agar powder(Manufacturer-HiMedia) in 1000ml distilled water.
2. Heat to boiling to dissolve the medium completely.
3. Mix well and distribute into test tubes.

4. Sterilize by autoclaving at 15 lbs pressure(121^0C) for 15 minutes .
5. Allow the medium to set in slopped form with butt about 1 inch long

Preparation Of Citrate Agar

1. Suspend 27.2 grams of Citrate agar powder(Manufacturer-HiMedia) in 1000ml distilled water.
2. Heat to boiling to dissolve the medium completely .
3. Sterilize by autoclaving at15lbs pressure (121^0C) for 15 minutes

Gram staining procedure

1. Make smear on slide, heat fix it.
2. Apply crystal violet for 1 minute.
3. Wash with water.
4. Add Gram's Iodine for 1 minute, wash with water.
5. Decolorize with spirit for 2-4 seconds.
6. Wash with water.
7. Add Saffranine stain for 30 seconds. Wash with water. See under oil immersion in microscope.

   Results: Gram positive- violet in color

   Gram negative- pink in color

ANNEXURE- VIII

MASTER CHART

CONTROL GROUP

SL.NO	Age	Sex	Address	Type of Specimen	Location	Type of bacteria isolated
1	48	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Klebsiella pneumonia, candida species, Peptostreptococcus species
2	48	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals
3	70	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Streptococcus species, candida species, micrococcus species
4	66	Male	Belgaum	Oral swab	Gingivobuccal sulcus	No growth seen
5	34	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Coagulase negative staphylococcus species
6	65	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals
7	56	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Pseudomonas species
8	38	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals
9	45	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Candida species
10	34	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals
11	48	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Streptococcus species, candida species,porphyromonas species.
12	58	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals, peptostreptococcus species.
13	56	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Klebsiella pneumonia, candida species, fusobacterium nucleatum.
14	51	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Staphylococcus viridans, peptostreptococcus species.

15	32	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Candida species, peptostrptococcus species.
16	53	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals
17	40	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals
18	32	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Escherichia coli, staphylococcus aureus, enterobacter species, prevotella species, peptostreptococcus species.
19	61	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals
20	38	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Pneumococci species
21	40	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Pneumococci species
22	42	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Pneumococci species
23	24	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Pneumococci species, Streptococci pneumoniae
24	42	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Klebsiella pneumonia
25	30	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Klebsiella pneumonia
26	55	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Klebsiella pneumonia
27	45	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Staphylococcus aureus
28	42	Male	Belgaum	Oral swab	Gingivobuccal sulcus	Oral commensals
29	42	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Klebsiella pneumonia
30	35	Female	Belgaum	Oral swab	Gingivobuccal sulcus	Streptococcus species

STUDY GROUP
(OSCC group)- Sample A- Surface of Oral Squamous Cell Carcinoma

SL.NO	Age	Sex	Address	Site/Location	Habit history		Type of bacteria isolated
						Surface	
						Aerobic	Anaerobic
1	60	Male	Hubli	Buccal mucosa	Tobacco chewing	Proteus species	0
2	61	Male	Belgaum	Alveolus	Tobacco chewing	Staphylococcus aureus	0
3	56	Female	Belgaum	Buccal mucosa	No habit history	Pseudomonas species	Peptostreptococcus anerobius
4	50	Male	Belgaum	Gingivobuccal sulcus	Tobacco chewing	0	Peptostreptococcus species, Fusobacterium species
5	38	Male	Hubli	Buccal mucosa	Tobacco chewing	Escherchia coli	0
6	65	Female	Belgaum	Tongue	No habit history	Klebsiella pneumonia	0
7	55	Male	Belgaum	Alveolus	Tobacco chewing	Pseudomonas species	0
8	48	Male	Belgaum	Buccal mucosa	No habit history	Pseudomonas species, Peptostreptococcus species	Pseudomonas species
9	65	Male	Belgaum	Tongue	Tobacco chewing	Streptococcus pneumoniae	0
10	60	Female	Belgaum	Buccal mucosa	No habit history	Pseudomonas species	0
11	42	Male	Hubli	Floor of the mouth	Tobacco chewing	Oral commensals	0
12	30	Male	Belgaum	Alveolus	Tobacco chewing	Oral commensals	0
13	49	Male	Belgaum	Buccal mucosa	Tobacco chewing	Oral commensals	0
14	54	Male	Belgaum	Tongue	Tobacco chewing	Pseudomonas species	0
15	49	Male	Belgaum	Buccal mucosa	Tobacco chewing	Oral commensals	0
16	60	Male	Belgaum	Buccal mucosa	Tobacco chewing	Pseudomonas species, klebsiella pneumonia	0
17	26	Female	Hubli	Tongue	Syndromic case- Tessier syndrome	0	0

18	52	Male	Hubli	Tongue			
19	64	Male	Belgaum	Floor of the mouth	Tobacco chewing	Candida species	0
20	60	Female	Belgaum	Tongue	No habit history	0	0
21	40	Male	Hubli	Buccal mucosa	Tobacco chewing	Pseudomonas species	0
22	52	Male	Hubli	Buccal mucosa	Tobacco chewing	Klebsiella pneumonia	0
23	59	Male	Belgaum	Alveolus & buccal vestibule	Tobacco chewing	0	Prevotella species
24	60	Male	Belgaum	Buccal mucosa	Tobacco chewing	0	0
25	27	Female	Belgaum	Gingivobuccal sulcus	No habit history	0	0
26	33	Male	Belgaum	Buccal mucosa	Supari and tobacco chewing	Streptococcus pyogens, enterococcus species	0
27	42	Male	Belgaum	Gingivobuccal sulcus	Tobacco chewing	Streptococcus pyogens	Porphyromonas species
28	55	Male	Belgaum	Buccal mucosa	Gutka chewing	Staphylococcus aureus	Porphyromonas species
29	60	Male	Belgaum	Buccal mucosa	Tobacco chewing	0	0
30	55	Male	Belgaum	Buccal mucosa and tongue	Tobacco chewing	0	0

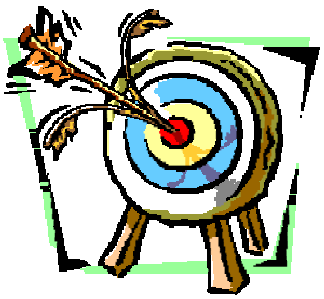
STUDY GROUP

(OSCC group)- Sample B- Deeper tissue of Oral Squamous Cell Carcinoma

SL.NO	Age	Sex	Address	Site/Location	Habit history		Type of bacteria isolated
						Deeper tissue	
						Aerobic	Anaerobic
1	60	Male	Hubli	Buccal mucosa	Tobacco chewing	Proteus species	0
2	61	Male	Belgaum	Alveolus	Tobacco chewing	0	0
3	56	Female	Belgaum	Buccal mucosa	No habit history	Candida species	Streptococcus species
4	50	Male	Belgaum	Gingivobuccal sulcus	Tobacco chewing	0	Peptostreptococcus species, Fusobacterium species
5	38	Male	Hubli	Buccal mucosa	Tobacco chewing	0	0
6	65	Female	Belgaum	Tongue	No habit history	0	0
7	55	Male	Belgaum	Alveolus	Tobacco chewing	Pseudomonas species	0
8	48	Male	Belgaum	Buccal mucosa	No habit history	0	0
9	65	Male	Belgaum	Tongue	Tobacco chewing	Streptococcus pneumoniae	0
10	60	Female	Belgaum	Buccal mucosa	No habit history	Klebsiella pneumonia, pseudomonas species	0
11		Male	Hubli	Floor of the mouth	Tobacco chewing	Candida species	0
12	30	Male	Belgaum	Alveolus	Tobacco chewing	0	0
13	49	Male	Belgaum	Buccal mucosa	Tobacco chewing	0	0
14	54	Male	Belgaum	Tongue	Tobacco chewing	0	0

15	49	Male	Belgaum	Buccal mucosa	Tobacco chewing	Oral commensals	0
16	60	Male	Belgaum	Buccal mucosa	Tobacco chewing	Prevotella species	0
17	26	Female	Hubli	Tongue	Syndromic case-Tessier syndrome	Candida species	0
18	52	Male	Hubli	Tongue			
19	64	Male	Belgaum	Floor of the mouth	Tobacco chewing	0	0
20	60	Female	Belgaum	Tongue	No habit history	Candida species	0
21		Male	Hubli	Buccal mucosa	Tobacco chewing	0	0
22		Male	Hubli	Buccal mucosa	Tobacco chewing	0	0
23	59	Male	Belgaum	Alveolus & buccal vestibule	Tobacco chewing	0	Porphyromonas species
24	60	Male	Belgaum	Buccal mucosa	Tobacco chewing	0	Bacteroides species
25	27	Female	Belgaum	Gingivobuccal sulcus	No habit history	0	Porphyromonas species
26	33	Male	Belgaum	Buccal mucosa	Supari and tobacco chewing	0	0
27	42	Male	Belgaum	Gingivobuccal sulcus	Tobacco chewing	0	Bacteroides species
28	55	Male	Belgaum	Buccal mucosa	Gutka chewing	Candida species	0
29		Male	Belgaum	Buccal mucosa	Tobacco chewing	0	0
30		Male	Belgaum	Buccal mucosa and tongue	Tobacco chewing	0	0

Introduction



Objectives

Review of Literature

Methodology

Results

Discussion

Conclusion & Summary

Limitations

Future Scope

Bibliography

Annexures