Original Research Article

Isolation and identification of various Candida species in potentially malignant disorders of oral cavity: a microbiological study

Abstract

Aim: The aim of this study was to find and compare various Candida species in the normal and potentially malignant disorders along with the evaluation of antifungal susceptibility of these species.

Study design: Cross-sectional study

Materials and Methods: 110 subjects were selected for the study comprising of 55 normal healthy individuals (control group) and 55 patients with potentially malignant disorders (test group). A saliva swab was taken from the normal healthy individuals and patients with potentially malignant disorders to check the presence of Candida on SDA media. A wedge shaped tissue sample from the representative site was surgically excised and sent for routine tissue processing for histopathological diagnosis. All the fungal colonies from SDA were subjected to further microbiological tests to check for the presence of various species of Candida.

Results: No fungus growth was found in saliva of healthy patients. Out of 55 patients of potentially malignant disorders, Candida growth was isolated by using the culture method, 30 patients (54.5%) showed positive growth on Sabouraud's Dextrose Agar (SDA) media, of which 23 were of diagnosed cases of leukoplakia and 7 oral submucous fibrosis. No fungus growth was seen in patients with oral lichen planus. There is positive association between the potentially malignant lesions and Candida and the p-value (.006) is statistically significant.

Conclusion: The results of the study showed that a positive association exists between the potentially malignant disorders and various Candida species and the p-value (.006) is statistically significant. The most prevalent Candida species was Candida albicans followed by Candida tropicalis, Candida glabrata, Candida Krusei

Keywords

Potentially malignant disorders, Candida, Microbiological tests.

Introduction

Cancer troubles all communities worldwide, being one of the ten most widespread cancers across the globe. Around 10 million people are detected with cancer and more than 6 million die due to the disease annually [1]. A significant proportion of oral squamous carcinomas spread from pre-existing premalignancies of the oral cavity. World Health Organization in 2007 suggested the term potentially malignant oral disorders for precancerous lesions and conditions. Oral potentially malignant disorders are defined as clinical presentations that carry a risk of cancer development in the oral cavity, whether in a clinically definable precursor lesion or in clinically normal mucosa [2]. If diagnosed and treated early they have better prognosis. Several risk factors such as tobacco, alcohol and viral infections play a significant role in potentially malignant disorders and cancer progression. Along with these; Candida has been considered an etiologic factor for potentially malignant disorders and oral cancer [3].

Candida comes under the phylum, Ascomycota and order, Saccharomycetales. It is a pathogenic dimorphic yeast-like fungus. There are approximately 200 organism species that come under the genus Candida. Species like Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida lusitaniae, Candida famata, Candida kefyr, Candida incospicua are the most frequently associated with human infection. Candida species are common members of the oral microflora and they can cause a range of opportunistic infections, candidiasis. Candida albicans is a predominant species that found in the oral cavity [3].

Due to the ever increasing numbers of immunocompromised patients in recent years, the prevalence of the disease precipitated by Candida species has expanded [4]. Apart from this, there are some local factors that give rise to oral candidiasis such as changes in salivary gland functions, use of antibiotics as well as corticosteroid drugs, diet rich in carbohydrates, dentures, alterations in oral epithelium, unrestricted use of tobacco products. Systemic factors that lead to oral candidiasis generally comprise the alterations in hormonal status, iron, folic acid, vitamin B12 deficiencies. Another important factors include malignant diseases, cytotoxic therapy and radiotherapy. Oral candidiasis may be recognized through discrete clinical signs and symptoms,

as acute pseudomembranous, acute atrophic candidiasis, chronic atrophic candidiasis, chronic hyperplastic candidiasis, glossitis as well as angular cheilitis [5].

Phenotypic switching of Candida is observed when they become pathogenic and invade the tissues. Yeast cells attach to oral epithelium through the cell wall proteins which are present on the surface of hyphal cells and promote their growth. Proposed mechanism for phenotypic switching is the secretion of degradation causing enzymes like proteases by the fungus which leads to the digestion of epithelial cell surface components. This permits the physical movement of hyphae into the host cells. Candida can induce the production of carcinogenic compounds like nitrosamines and N-nitrosobenzylmethylamine. Some strains of Candida have high nitrosation potential that give rise to more advanced precancerous alterations. Yeast cells then extend from mucosal surface to deeper epithelial cell layers and deposit the nitrosamines to deeper layers. Some strains of Candida albicans play an important role in the progression of dysplasia. Carcinogenic compounds can bind to DNA and cause miscoding with DNA replication, which results in formation of an oncogene as well as initiates cancer progression. Most common potentially malignant disorders associated with Candida are leukoplakia, erythroplakia, oral submucous fibrosis as well as lichen planus [3].

The present study was undertaken to investigate the various species of Candida (Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata, Candida krusei) in potentially malignant disorders and to correlate their presence in normal healthy individuals.

Material and methods

The study had been approved by the Ethical Committee ((PUIEC/2019/149/A-1/01/03) of the Dental Institute. The study was conducted in the department of Oral and Maxillofacial Pathology in collaboration with Department of Oral Medicine & Radiology and Department of Microbiology. A total number of 110 individuals were included, of which 55 individuals clinically diagnosed as OPMDs such as leukoplakia, OSMF and oral lichen planus [Figure 1,2,3] and 55 individuals were normal healthy controls. Patients on topical or systemic antifungal therapy, those presenting with any systemic illness especially diabetes and currently pregnant or lactating individuals were excluded from the study. Each participant was included after obtaining the written consent. Detailed oral examination of all the individuals were carried out using

diagnostic instruments. Saliva sample using a cotton swab was obtained from the representative site of OPMDs, control group patients.Brain heart infusion broth (BHIB) was used as transport media for the transportation of saliva to the microbiology laboratory.Subject with OPMDs was sent for biopsy sampling for confirmatory diagnosis.

Saliva sample and microbiological parameters-Saliva was collected using a sterile cotton swab from the representative site of OPMDs, control group patients. Brain heart infusion broth (BHIB) was used as transport media for the transportation of saliva to the microbiology laboratory. Saliva sample was inoculated on Sabouraud dextrose agar. The sample was streaked using inoculating loop and incubated at37^0 C for 48 hours. The growth appeared in 48 hours as cream/white colored, smooth and pasty colonies [Figure 4].

Lactophenol cotton blue staining was used for the detection of fungal elements in clinical sample. A drop of Lactophenol cotton blue reagent was placed on a clean and dry slide. Nichrome inoculating wire was used to spread the fungal culture into a thin preparation. Then the coverslip was placed on the thin preparation. Using light microscope (Nikon Light Microscope, Eclipse 50i, Tokyo, Japan) the slides were evaluated for presence of fungal elements in patients of potentially malignant disorders and normal healthy individuals [Figure 5].

Tetrazolium reduction medium was used to differentiate various Candida species. Colonies of yeast were picked off from SDA and plated out on to tetrazolium reduction medium incubated at 37^0 C. Tetrazolium plates were examined for growth and colour changes after 24 hours [Figure 6].

Later, the confirmatory biochemical test such as sugar fermentation test was carried out for Isolated Candida species (Candida albicans, Candida tropicalis, Candida glabrata and Candida Krusei).Liquid media (Andrade's peptone water) and 5 sugars (Glucose, galactose, maltose, lactose, trehalose) were poured in different test tubes containing Durham's tube and sterilized by autoclaving. Each tube was inoculated with 0.1 ml of inoculum. The tubes were incubated at 25^0 C for up to 1 week and were examined at every 24 hours interval for the production of acid (pink color) and gas (in Durham's tube).Production of gas in the tube was taken as fermentation positive while only acid production was taken as carbohydrate assimilation [Figure 7].

Tissue sample and histopathological staining-Tissue was surgically excised from the representative area from all subjects enrolled in test group for confirmation of diagnosis of pathology. For OPMD cases, a wedge-shaped biopsy sample was taken with a 1 mm margin to the depth of submucosa. The excised samples were immediately fixed in 10% neutral buffered formalin for 24 hours followed by routine histopathological processing and embedded in paraffin wax.

The rough cutting was done for exposure of the samples embedded in paraffin blocks using semi-automatic microtome (LEICA RM2245, Ontario, Canada) at 20 microns. Further, finer sections of 2.5- 3.0 microns were prepared and mounted on slides (Blue Star) coated with egg albumin for routine hematoxylin-eosin staining for the demonstration of potentially malignant disorders and Periodic acid Schiff staining for the demonstration of fungal elements in potentially malignant disorders. Using light microscope (Nikon Light Microscope, Eclipse 50i, Tokyo, Japan) the slides were evaluated for presence of histopathological features of oral potentially malignant disorders and presence of fungal hyphae in patients of potentially malignant disorders.

Results

In the present study, 110 participants were equally divided into two groups- 55 Potentially malignant lesions (Group I) and 55 healthy controls (Group II). Group I included 35 patients (63%) of oral leukoplakia, 13 patients (24%) of oral submucous fibrosis and 7 patients (13%) of oral lichen planus [Graph 1][Table 1]. The clinical diagnosis of potentially malignant disorders was confirmed by histopathology. PAS staining was performed to confirm the presence of fungal elements in potentially malignant lesions. In Group I, no fungal element was found by PAS staining (0.0%)[Graph 2][Table 2]. No fungus growth was found in saliva of healthy patients. Candida growth was isolated by using the culture method. 30 out of 55 patients (54.5%) with potentially malignant disorders showed positive growth on Sabouraud's Dextrose Agar (SDA) media, of which 23 were diagnosed cases of leukoplakia and 7 oral submucous fibrosis. No fungus growth was seen in patients with oral lichen planus. A positive association between the potentially malignant lesions and Candida was found with a statistically significant p-value (0.006) [Graph 3][Table 3].

All positive cases for fungal growth were stained for Lectophenol cotton blue staining. All 30 cases (100%) reveal the presence of opaque yeast cells which appear oval, dark and polymorphic [Figure 5][Graph 4][Table 4].

Isolation of Candida species was carried out for the positive cases on SDA media using Tetrazolium reduction medium. Candida albicans were isolated from all 30 patients. Non-albicans Candida species (Candida tropicalis, Candida glabrata and Candida krusei) were isolated from 18 of the 30 positive patients studied [Figure 6][Graph 5][Table 5]. (13 out of 23 patients of oral leukoplakia and 5 out of 8 patients of oral submucous fibrosis)

Confirmatory biochemical test such as sugar fermentation test was carried out for the isolated Candida species (Candida albicans, Candida tropicalis, Candida glabrata and Candida krusei). Isolates of Candida albicans, Candida tropicalis as well as Candida glabrata showed carbohydrate assimilation and fermentation for glucose, carbohydrate assimilation for galactose, but no carbohydrate assimilation as well as fermentation was seen in lactose, carbohydrate assimilation and fermentation for maltose and carbohydrate assimilation for trehalose. Isolates of Candida krusei showed carbohydrate assimilation and fermentation for glucose, carbohydrate assimilation for galactose, no carbohydrate assimilation as well as fermentation was seen in lactose, carbohydrate assimilation for maltose and trehalose [Figure 7].

Discussion

Oral cancer is the most common neoplasm among all malignancies. The survival rates of oral cancer are low, and they differ among ethnicities and age groups. Oral potentially malignant disorders (OPMD) are chronic conditions that can carry a risk of cancer development in the oral cavity. Many etiological factors are related to the development of OPMDs as well as oral squamous cell carcinoma. However, the main associated factors are tobacco and alcohol. Along with these, Candida has been considered an etiologic factor for potentially malignant disorders and oral cancer. Therefore, early diagnosis through screening as well as appropriate treatment can reduce the oral cancer burden globally [2].

Candida is a yeast- like fungus. Candida is derived from Latin word toga Candida, referring to the white toga (robe) worn by candidates for the Senate of the ancient Roman republic. The specific epithet albicans also comes from Latin, albicare meaning "to whiten". These names refer

to the generally white appearance of Candida species on culture media. The most common species of Candida is the C. albicans which is commonly present in both yeast and hyphae forms. C. albicans can form the pathogenic commensal which colonizes, penetrates and damages the host tissues. This activity is attributed to the factors such as imbalance between C. albicans virulence factors and host defenses as well as specific defects in the immune system. Several cell surface proteins known as adhesion recognizing host molecules are present which interact with a wide variety of host proteins, particularly some extracellular matrix components like fibronectin, laminin and collagen. Plasmatic components, such as fibrinogen, iC3b and C3d, have also been proposed as mediators of adherence of C. albicans. Candida can then produce carcinogenic compounds, such as nitrosamines, N- nitrosobenzyl methylamine. Strains with high nitrosation potential have been isolated from lesions with more advanced precancerous changes. These compounds have the ability to bind with DNA to form adducts due to which the miscoding or irregularities with DNA replication occurs leading to oncogene formation and cancer initiation [3].

In the present study, we attempted to isolate and identify the various Candida species in potentially malignant disorders of oral cavity. The clinical diagnosis of potentially malignant disorders was confirmed histopathologically. For microbiological purposes, saliva samples were collected using sterile swab. Similar saliva collection technique was employed by Roy et al from patients of potentially malignant disorders and oral squamous cell carcinoma [6].

The collected swab sample was inoculated primarily on SDA medium followed by tertrazolium reduction medium for speciation. A study conducted by Sarkar et al included 40 patients with oral leukoplakia and 21 controls, using SDA as a primary media followed by germ tube and corn meal tests which identify only Candida albicans and Candida dubliniensis and are unable to identify other Candida species. They concluded that nineteen cases of leukoplakia showed Candida on direct smears, compared to 3 controls. Eighteen cases and one control showed growth of Candida on culture. Non-homogenous leukoplakia showed a higher positivity rate on microscopy and culture than homogenous lesions. All these correlations were statistically significant [7]. In our study, no fungal growth was found in the normal control group which is in accordance with the study conducted by Saigal et al and Gupta et al.

In our study, 65.7% cases of oral leukoplakia, 53.8% cases of oral submucous fibrosis showed positive growth on Sabouraud's Dextrose Agar (SDA) media. No Candidal growth (0.0%) was seen in patients of oral lichen planus. A study conducted by Saigal et al revealed that 53.3% cases of leukoplakia, 20% cases of oral submucous fibrosis showed fungus growth whereas in Oral squamous cell carcinoma 66.6% cases showed fungal growth [1]. In our study, no Candidal growth was seen in patients of oral lichen planus, a finding in contradiction to the study conducted by Galle et al where they found fungus growth in 45.4% cases of oral lichen planus [4].

All positive cases for fungal growth were stained for Lectophenol cotton blue staining. All 30 cases (100%) reveal the presence of opaque yeast cells which appeared oval, dark and polymorphic. The probability 'p' value was significant ($p<0.0001$) on using Chi-square test.

PAS staining is useful for the demonstration of candidal hyphae as well as yeast. By using this method, fungal elements generally appear dark blue or red/purple. In our study, however, no fungal element was disclosed by PAS staining in any of the cases of study and control groups. Recently, other fluorochrome stains such as Calcofluor white (CFW) and Acridine orange are commercially available to demonstrate fungal elements in tissue sections, smears and fresh preparations, rapidly and simply. Kumar et al compared CFW and PAS stain and concluded that CFW detects more Candida microorganisms and is more efficient than PAS staining [8]. Contrastingly, Galle et al found that out of 48 cases of oral squamous cell carcinoma 18 cases (37.5%) showed the presence of fungal elements by PAS staining whereas out of 55 cases of potentially malignant lesions, 15 cases (27.27%) disclosed the fungal elements [4]. In the present study, Tetrazolium reduction medium was used to differentiate various Candida species. Tetrazolium is reduced in different gradients by various species of Candida to produce different colours depending on the species. The most prevalent Candida species identified under Tetrazolium reduction medium was Candida albicans (cream glistening) followed by Candida tropicalis (dark maroon red), Candida glabrata (pale pink), Candida krusei (dry pink). Candida albicans was isolated from 23 patients of oral leukoplakia and 7 patients of oral submucous fibrosis. Non-albicans Candida species (Candida tropicalis, Candida glabrata and Candida Krusei) were isolated from 13 patients (37.1%) of oral leukoplakia as well as 5 patients (38.5%) of oral submucous fibrosis. The probability 'p' value was significant ($p<0.0001$) on using Chi-

square test. A study conducted by Maire J et al included 1822 yeast isolates from the laboratory and plated on to the tetrazolium reduction medium and incubated at 370 C. They found 7 different coloured isolates (C. albicans, C. stellatoidea, C. tropicalis, C. pseudotropicalis, C. krusei, C. parapsilosis, C. guilliermondii) of Candida on this medium. Another study conducted by Denny et al from London in 1968 found tetrazolium reduction media a rapid, relatively accurate and simple means of differentiating Candida species from other yeasts and being less expensive than other secondary media such as CHROMagar.

A study conducted by Roy et al where CHROMagar was used for the speciation, revealed that Candida species was present in 5 (20%) individuals from control group, 12 (40%) cases of OPMDs and 31 (77%) cases of OSCC and absent in 20 (80%) individuals from control group, 18 (60%) cases of OPMDs and 9 (23%) cases of OSCC [7].

Confirmation of infection by Candida species requires laboratory isolation and identification. Along with conventional tests, biochemical tests such as sugar fermentation tests and sugar assimilation tests are being increasingly used in clinical microbiology laboratories worldwide for the speciation of Candida isolates. In the present study, isolates of Candida (Candida albicans, Candida tropicalis, Candida glabrata and Candida krusei) were confirmed by biochemical tests such as sugar fermentation tests. Isolates of Candida albicans, Candida tropicalis as well as Candida glabrata showed carbohydrate assimilation and fermentation for glucose, carbohydrate assimilation for galactose, but no carbohydrate assimilation as well as fermentation were seen in lactose, positive carbohydrate assimilation and fermentation for maltose and carbohydrate assimilation for trehalose were also found. Isolates of Candida Krusei showed carbohydrate assimilation and fermentation for glucose, carbohydrate assimilation for galactose, but no carbohydrate assimilation as well as fermentation were seen in lactose, positive carbohydrate assimilation for maltose and trehalose were found.

Potentially malignant disorders associated with Candida should be treated with great caution as it shows a higher rate of malignant transformation [3]. Antifungal susceptibility patterns of infectious fungi are a crucial determinant that contributes to the outcome of patients. While the incidence of Candida infections in potentially malignant disorders and oral cancer is increasing, the choice of suitable antifungal agents is limited due to the resistance of some species to several antifungal agents. The activities of antifungal agents are important therapeutic options to control

infections caused by Candida albicans and non-albicans species. The appropriate treatments are dependent on the immune status and underlying diseases of patients, the specific Candida species involved and its susceptibility pattern to antifungal agents. A special focus should be given to the therapeutic aspect of these fungal infections.

Our study concluded that, there is positive association of various species of Candida in potentially malignant disorders such as oral leukoplakia and oral submucous fibrosis but no fungal association was seen in the patients of oral lichen planus and the p-value (.006) is statistically significant. As the oral mucosa is compromised in patients of potentially malignant disorders and presence of Candida species may play an important role in the development of potentially malignant disorders and their transformation in oral cancer by means of endogenous nitrosamine production.

The diagnosis of oral potentially malignant disorders and oral cancer is fundamentally clinical. Microbiological methods of diagnosis should be used for confirmation of Candida in patients of potentially malignant disorders and oral cancer.

Conclusion

Early diagnosis of potentially malignant disorders greatly decreases the risk of oral cancer. Primary prevention which involves reducing the exposure to tobacco, alcohol and betel quid has been shown to be effective in reducing the incidence of oral cancer. Secondary prevention involves screening for the early detection of oral cancer. Oral cancer screening can take many forms. Clinical examination and biopsy allow the early detection of potentially malignant disorders and early oral cancers. Histological methods or stains may disclose the fungal elements in tissue specimens, which may indicate that the yeast have invaded the tissue. More number of studies should be done in the field of fluorochrome stains to judge the sensitivity as well as specificity of these stains. The frequent isolation of various species of Candida in patients of potentially malignant disorders and oral squamous cell carcinoma indicates a need for effective treatment of various opportunistic infections as they can play a definite role in carcinogenesis and can lead to progression of the disease to be potentially lethal. The present study was carried out with the aim to find and compare various Candida species in the normal healthy individuals and potentially malignant disorders. A positive association exists between the potentially

malignant disorders and various Candida species and the p-value (.006) is statistically significant.

Consent

As per international standard and university standard, patient's written consent has been collected and preserved by the author(s).

Ethical approval

The study had been approved by the Ethical Committee ((PUIEC/2019/149/A-1/01/03) of the Dental Institute.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Figure 1. Patient presenting with reddish white patch on the right and left buccal mucosa

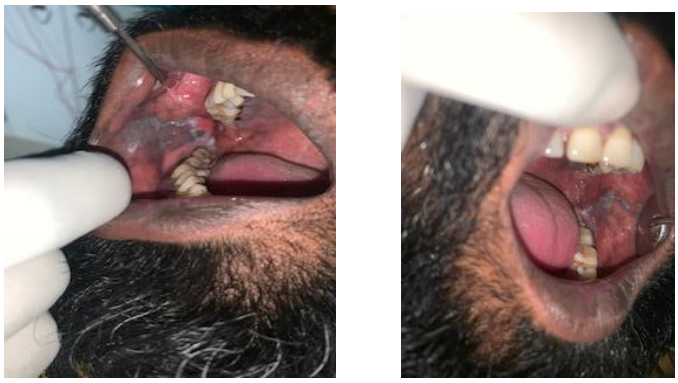


Figure 2. Patient presenting with blanched appearance of right and left buccal mucosa and restricted mouth opening

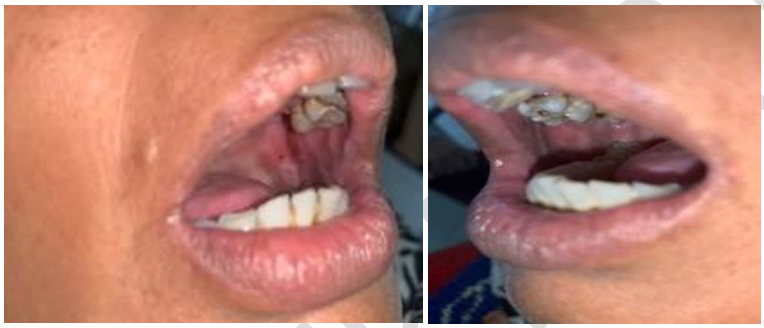


Figure 3. Patient presenting with white patch over the buccal mucosa and dorsal surface of tongue

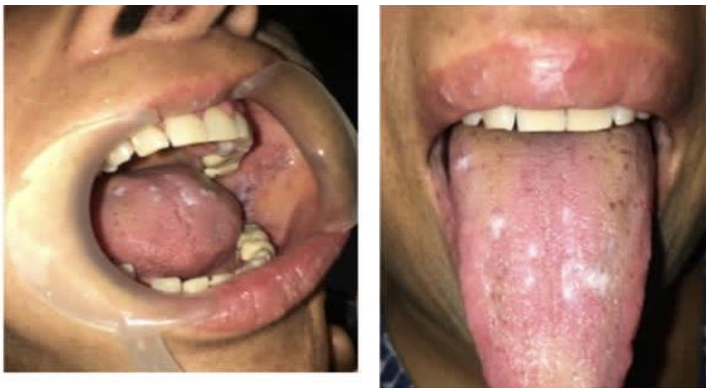


Figure 4. Sabouraud's Dextrose Agar media showing white creamy pasty colonies representative of Candida

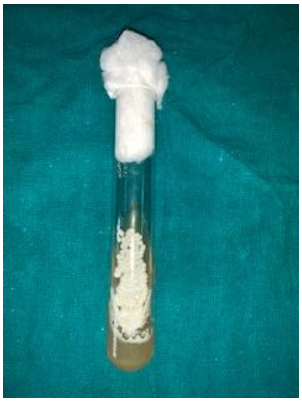


Figure 5. Photomicrograph showing yeast cell after LCB staining (20X)

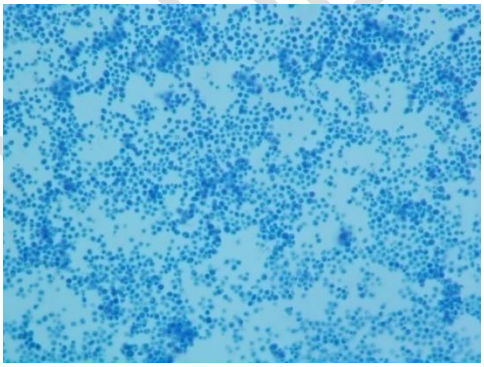


Figure 6. Tetrazolium reduction medium showing cream color colonies representative of C. albicans, Pale pink colonies representative of C. glabrata, Pink dry colonies representative of C. krusei, Dark maroon colonies representative of C. tropicalis.

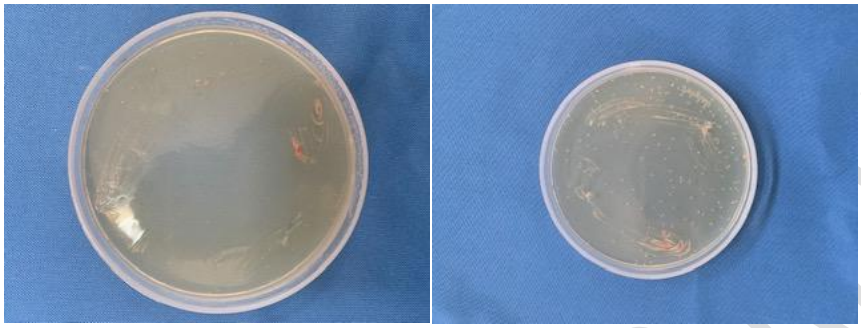
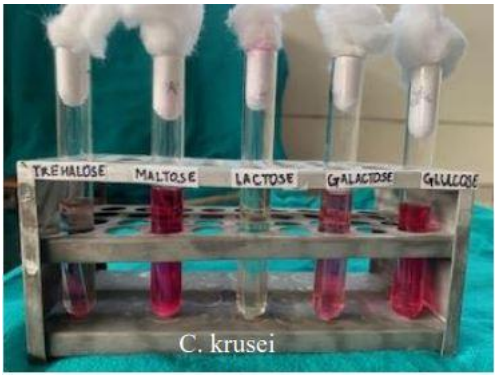
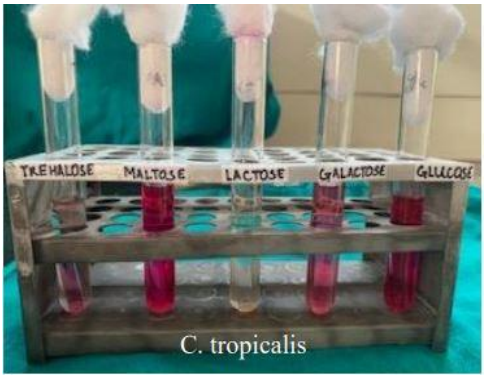


Figure 7. Sugar fermentation test positive for C. albicans, C. glabrata, C. krusei, C. tropicalis

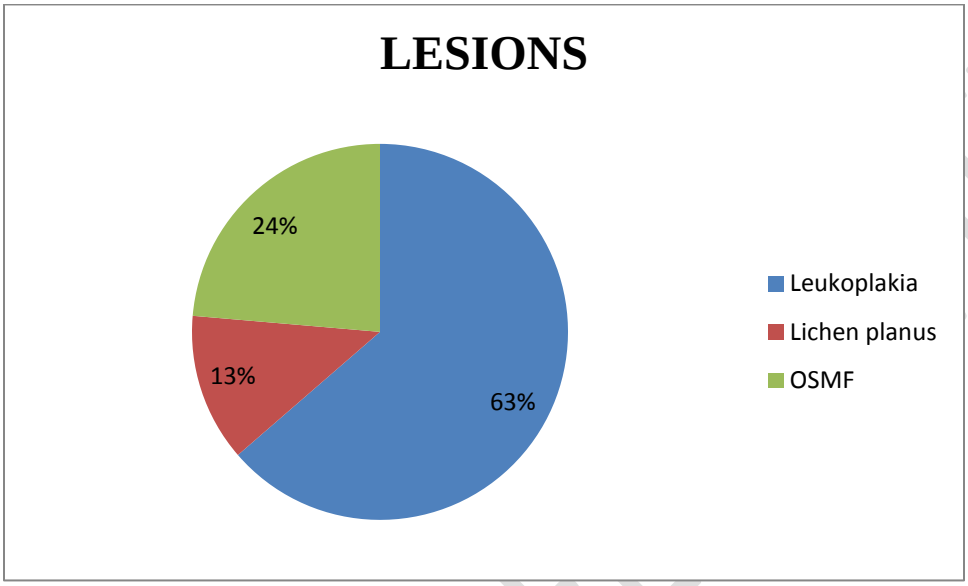


C. krusei

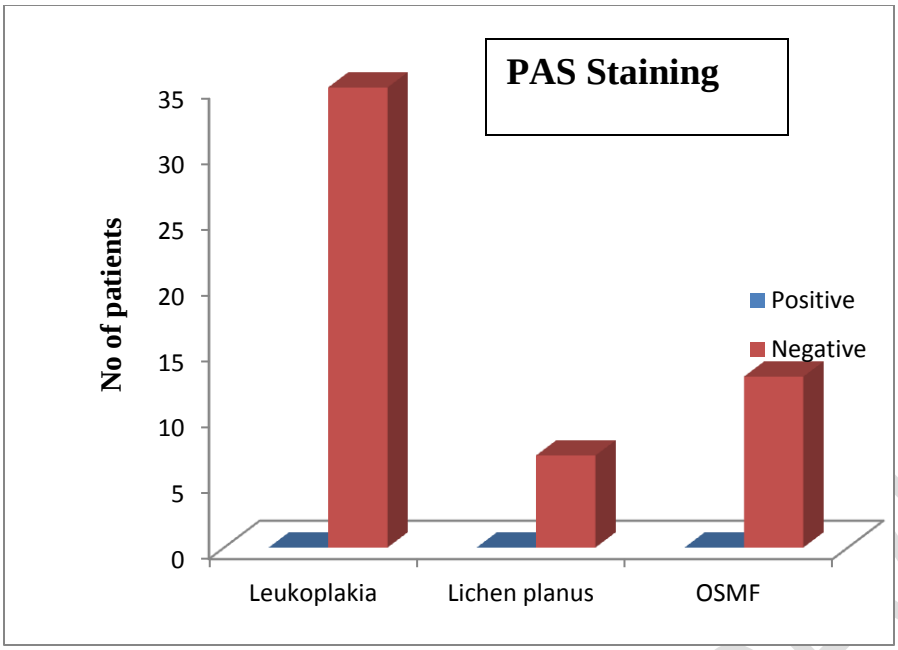


C. tropicalis

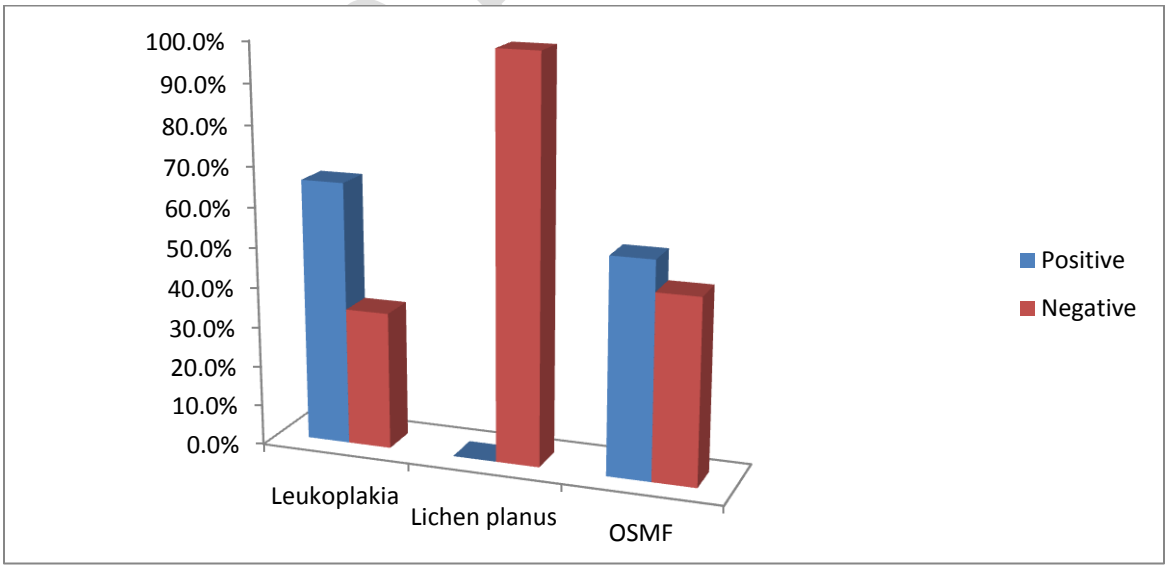
Graph 1. Percentage of Leukoplakia, OSMF, Lichen planus in test group has been shown.



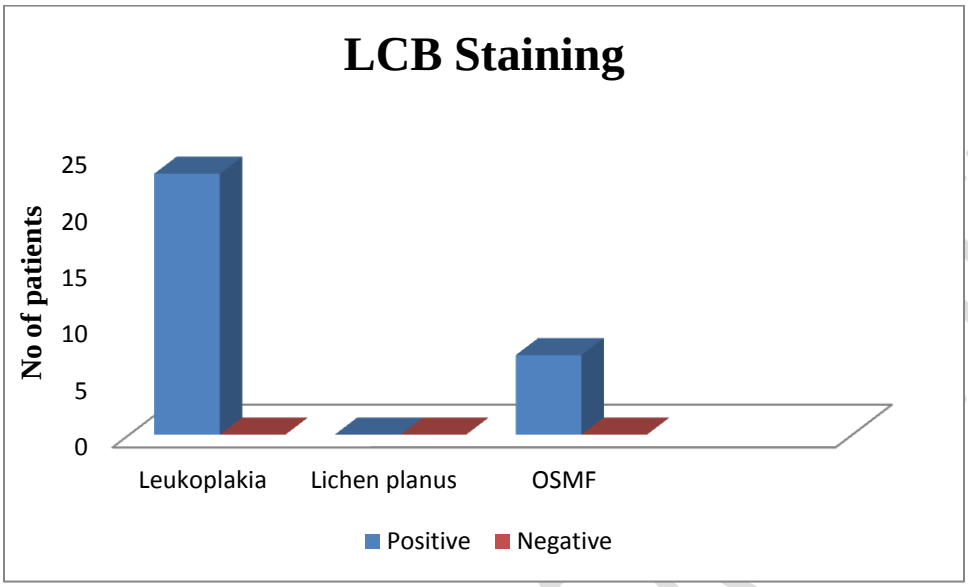
Graph 2. Fungal elements disclosed by PAS staining in test group



Graph 3. Percentage of fungal growth with Sabouraud's Dextrose Agar media in potentially malignant disorders



Graph 4. Fungal elements disclosed by LCB staining in test group



Graph 5. Percentage of different species of Candida in potentially malignant disorders

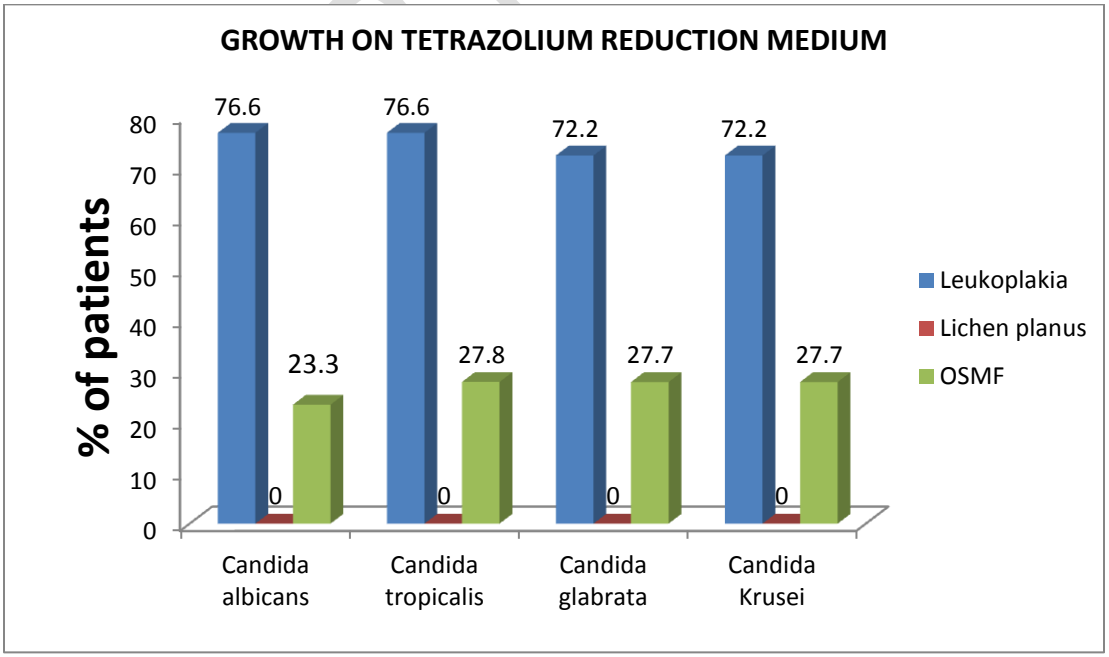


Table 1. Frequency and percentage of potentially malignant disorders in test group

LESIONS			p-value
	Frequency	Percent	
Leukoplakia	35	63.6	.0001**
Lichen planus	7	12.7	
OSMF	13	23.6	
Total	55	100.0	

Table 2. Frequency and percentage of fungal elements disclosed by PAS staining in test group

PAS STAINING			p-value
	Frequency	Percent	
Positive	0	0.0	.0001**
Negative	55	100.0	
Total	55	100.0	

Table 3. Evaluation of Candida on SDA media in potentially malignant disorders

		CANDIDA GROWTH ON SDA		Total	Chi-Square	p-value
		Positive	Negative			
LESIONS	Leukoplakia	23	12	35	10.164	.006**
		65.7%	34.3%	100.0%		
	Lichen planus	0	7	7		
		0.0%	100.0%	100.0%		
	OSMF	7	6	13		
		53.8%	46.2%	100.0%		
Total		30	25	55		
		54.5%	45.5%	100.0%		

Table 4: Frequency and percentage of fungal elements disclosed by LCB staining in test group

LCB STAINING			p-value
	Frequency	Percent	
Positive	30	100.0	.0001**
Negative	0	0.0	
Total	30	100.0	

Table 5. Evaluation of different species of Candida on TRM media in potentially malignant disorders

		GROWTH ON TETRAZOLIUM REDUCTION MEDIUM		Total	Chi-Square	p-value
		Positive	Negative			
LESIONS	Leukoplakia	13	22	35	3.91	.142
		37.1%	62.9%	100.0%		
	Lichen planus	0	7	7		
		0.0%	100.0%	100.0%		
	OSMF	5	8	13		
		38.5%	61.5%	100.0%		
Total		18	37	55		
		32.7%	67.3%	100.0%		