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RESEARCH ARTICLE

ASSESSING THE PREVALENCE AND CORRELATION OF CANDIDA SPECIES IN LEUKOPLAKIA AND ORAL CANCER: A COMPARATIVE STUDY

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ABSTRACT

Background: Head and neck cancer is a major problem that occurs in Asia. Increasing incidence and lack of improvement in prognosis of oral cancer and pre cancer in Indian population necessitates in depth probing of various etiological and contributory factors for its early diagnosis and prognosis. Various studies have focused supporting an association between Candida species and oral neoplasia. Objective – This study was conducted to assess the prevalence and correlation of Candida organisms in leukoplakia, oral cancer and healthy control group**.** ***Method*****:** A comparative study was carried out in the Department of Oral Medicine and Radiology, which include total 63 patients based on inclusion and exclusion criteria 21 clinically and histopathologically diagnosed oral cancer patients and 21 leukoplakia patients and 21 subjects with no clinical lesion were included in the study. The biopsy specimen processed for routine histopathologic examination and PAS staining. Whole saliva sample collected by spitting method was incubated in Sabouraud's dextrose agar by lawn plating technique. and colonies were counted for Candida species. ***Results***: In present study we found positive correlation of Candida species in oral cancer and leukoplakia patients. The distribution of mean candida colonies in oral cancer group was significantly higher compared to the leukoplakia and control groups (p-value <0.001 for both). The distribution of prevalence of presence of candida colonies differ significantly between oral cancer group and normal group as well as between leukoplakia and normal group (p-value<0.05 for both). ***Conclusion***: Present study indicates positive role of candida in oral carcinogenesis.

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INTRODUCTION

Cancer afflicts all communities worldwide. Approximately 10 million people are diagnosed with cancer out of which 6 million die. In India most common and frequent cause related to cancer death is oral cancer. Many viruses and fungi have been implicated in aetiology of oral cancer. The oral cavity harbors hundred of different microbial species and Candida is the most common fungal pathogen in humans, which exists as a commensal inhabitant of mucosal surfaces in most healthy individuals. However alterations of host or environment can lead to overgrowth of fungus and infection to the host[1]. In recent years, many researchers have focused on studies supporting an association between Candida species and oral neoplasia. Interest in the study of oral candidiasis has markedly increased not only because of its association with human immunodeficiency virus (HIV) infection, but also due to its relation with precancerous and cancerous lesions of the oral mucosa. These lesions belong to the wide group of leukoplakia and oral cancer. Candida leukoplakia, if untreated, has a significant rate of malignant transformation when compared with non-Candida leukoplakia. This high rate of malignant change in Candida leukoplakia is strong evidence for the fungi being involved in neoplastic change[2].

Candida species are common members of oral micro flora and are generally regarded as being commensals. Presence of Candida along with epithelial changes such as atrophy, hyperplasia and dysplasia may compromise the mucosal barrier and facilitate Candida invasion thereby giving rise to a range of opportunistic infections referred to as candidiasis[3]. A rare Candida albicans biotype having a high nitrosation potential has been isolated from non homogeneous leukoplakia, suggesting endogenous production of carcinogenic nitrosamines. Candida albicans isolated from potentially malignant disorders may also produce mutagenic amounts of acetaldehyde. Further, a biological injection phenomenon has been proposed by which hyphae may serve as tracts through which carcinogens travel deep into the epithelium even though Candida affects only the superficial layers initially[4]. With increasing importance of candidiasis, there is need for identification of Candida in potentially malignant lesion. Present study was undertaken to evaluate the prevalence and identify the correlation of Candida in leukoplakia and oral cancer.

MATERIALS AND METHODS

After obtaining permission from The Institutional research board and Institutional Ethical Committee, data collection

procedure started in the Department of Oral Medicine and Radiology. Based on inclusion and exclusion criteria 21 clinically and histopathologically diagnosed oral cancer patients and 21 leukoplakia patients and 21 subjects with no clinical lesion were included in the study. All the participants were explained the need and design of the study and the need for undergoing a thorough clinical examination, biopsy and saliva sample investigations at the start of the study. A biopsy was performed for leukoplakia and oral cancer and normal control group under local anaesthesia. The biopsy specimen processed for routine histopathologic examination. Slides were assessed using haematoxylin and eosin staining and the presence of yeast was assessed utilizing periodic acid Schiff (PAS) stain (Figure 1). The subjects who are clinicopathologiaclly confirmed with leukoplakia and oral cancer had considered for further salivary investigation. Biopsy from normal control group was taken from the patients who are undergoing any oral surgical procedures of mucosa eg – implants, crown lengthening procedures, orthodontic exposure of teeth etc. was considered for salivary investigation. Only those patients, who gave a signed informed consent on an institutionally approved document, participated in the study. The salivary investigation carried out by collecting whole saliva sample which involved rinsing of the mouth with sterile saline. Saliva sample collected with spitting method in sterile container and stored at -20 Celsius and then transferred to the laboratory in ice pack. Each rinse was centrifuged (2,000 × g; 10 min), the supernatant was removed, and the deposit was re-suspended in 1 ml of phosphate-buffered saline (PBS), serial dilution of the sample up to 10^5 done and a portion (50 µl) of the concentrate was inoculated onto Sabouraud's dextrose agar with a cotton swab by lawn plating technique or spread plate's technique prior to incubation at 37°C for 48 hr (Figure 2). Suspected Candida colonies were sub-cultured onto plates of CROM-agar Candida for isolation of multiple yeast species(Figure 3). After incubation observe and count the colonies forming unit of Candida species. (Kumari S, Ichhpujani et al)

RESULTS

The present study evaluates the prevalence and correlation of candida with normal controls, leukoplakia and oral cancer patients. Our study included total 63 patients, 21 in each group as per inclusion criteria age ranging from 15 years to 74 years with Mean age found to be 43 years. The distribution of mean age in oral cancer group was significantly higher compared to the leukoplakia and control group (p-value<0.001 for both). (Table 1). Out of 63 patients, 26 patients come under below 40 years of age in which 22 patient (84.6%) had candida colonies and in 4 patients (15.4%) candida colonies were absent in saliva. 16 patients fall in between 40 to 50 year of age group in which 14 were (87.5%) positive for presence of candida colonies and in 2 patients (12.5%) candida colonies were absent in saliva. 21 patients were above 50 years of age, all were positive for candida colonies in saliva. Out of 63 patients 50 (58.2%) were male and 13(20.7%) were female. Male predominance is seen all the groups. Out of 50 males, 45 patients (90%) had candida colonies in saliva and 5 patients (10%) had no candida colonies. Out of 13 females, 12 patients (92.3%) had candida colonies in saliva. In our study we found smoking and smokeless form of tobacco and alcohol as known factor for causing oral cancer. Out of 63 patients, 54 (85.8%) patients were habituates. All the 21 patients in oral cancer group and leukoplakia were habituates. Out of 21 oral cancer patients 10 (47.6%) come under smoking tobacco habit and 11 (52.4%) were using smokeless tobacco and in leukoplakia group 14 patients (66.6%) were habituated of smokeless tobacco and 7 patients (33.7%) habituated of smoking. In control group 12 volunteers (57.1%) were habituates of smokeless tobacco and 9 volunteers (42.9 %) were non habituates (**Table 2).**

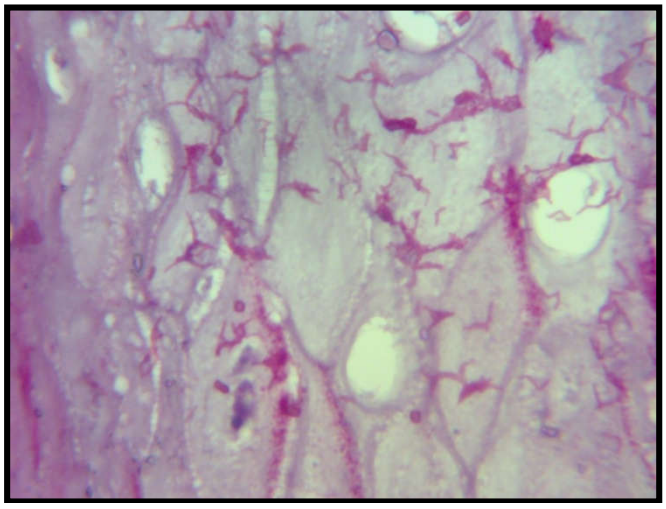


Figure 1. Magenta color Candida hyphae after PAS

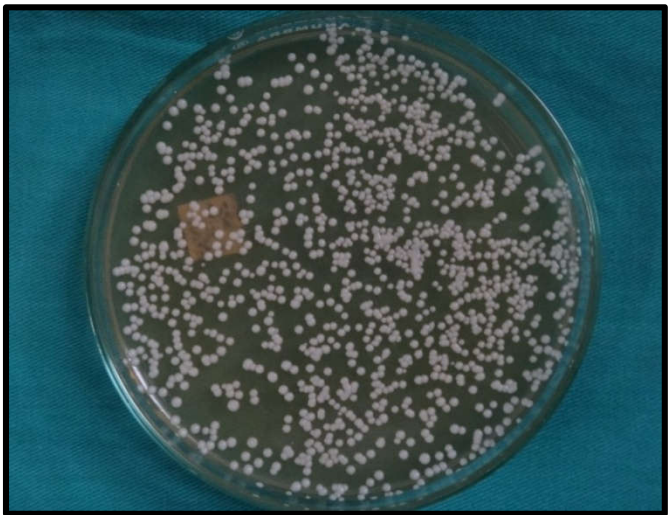


Figure 2. Colonies of fungus on Sabouraud's agar after incubation

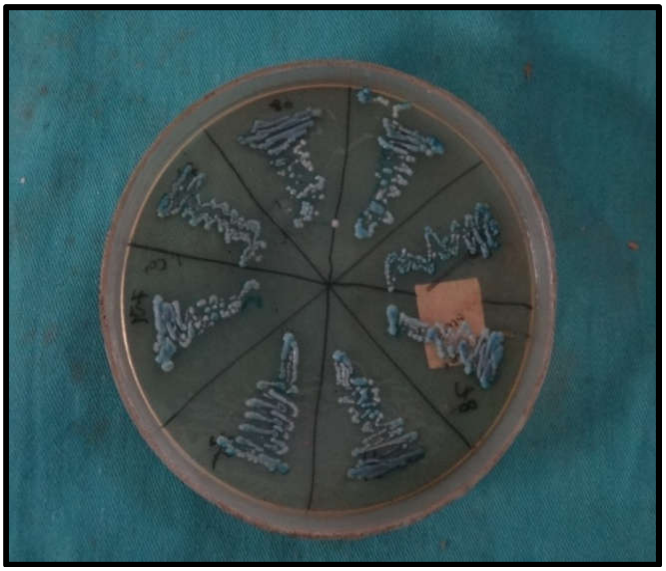


Figure 3. Green color growth on Hi Chrome agar plate after 48 hours incubation

Table 1. Distribution of mean age across three study groups

	Group 1 (n=21) [OSCC]		Group 2 (n=21) [LEUKO]		Group 3 (n=21) [NORMAL]		P-values (Inter-Group)		
	Mean	SD	Mean	SD	Mean	SD	Group 1 v Group 2	Group 1 v Group 3	Group 2 v Group3
Age (years)	55.6	9.0	41.6	13.6	35.1	11.9	0.001***	0.001***	0.179NS

Values are mean and Standard deviation (SD). p values by one-way analysis of variance (ANOVA) with Post-Hoc Tukey's test for multiple group comparisons. p value<0.05 is considered to be statistically significant. *p value<0.05, ***p value<0.001, NS-Statistically Non-Significant.

Table 2: Distribution of substance abuse (habits) across three study groups

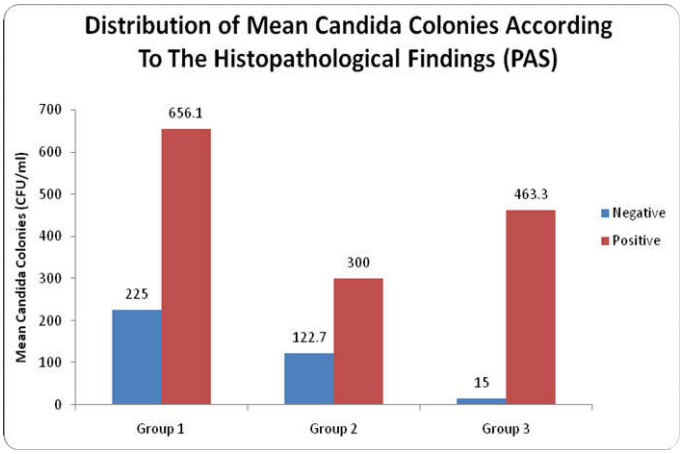
	Group 1 (n=21) [OSCC]		Group 2 (n=21) [LEUKO]		Group 3 (n=21) [NORMAL]		p values (Inter-Group)		
Habits	N	%	n	%	N	%	Group 1 v Group 2	Group 1 v Group 3	Group 2 v Group 3
Nil	0	0.0	0	0.0	9	42.9	0.999NS	0.003**	0.003**
A.Smoking B. Non smoking	10	47.6	7	33.3	0	0.0			
Tobacco /Gutkha	10	47.6	14	66.7	10	47.6			
Mishri	1	4.8	0	0.0	2	9.5			
Total	21	100.0	21	100.0	21	100.0			

Values are n (% of cases). p values by Chi-Square test with Post-Hoc Tukey's test for multiple group comparisons. p value<0.05 is considered to be statistically significant. *p value<0.05, **p value<0.01, NS-Non-Significant.

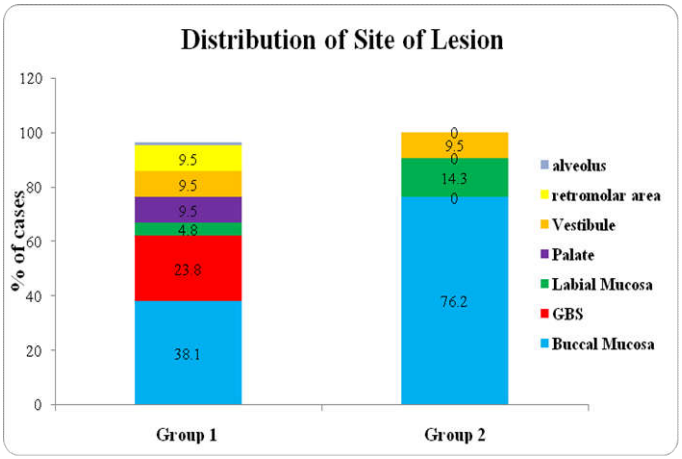
Table 3. Distribution of mean Candida colonies across three study groups

	Group 1 (n=21) [OSCC]		Group 2 (n=21) [LEUKO]		Group 3 (n=21) [NORMAL]		p values (Inter-Group)		
	Mean	SD	Mean	SD	Mean	SD	Group 1 v Group 2	Group 1 v Group 3	Group 2 v Group 3
Candida Colonies (CFU/ml)	613.0	385.9	190.2	116.7	79.0	210.8	0.001***	0.001***	0.355NS

Values are mean and Standard deviation (SD). p values by one-way analysis of variance (ANOVA) with Post-Hoc Tukey's test for multiple group comparisons. p value<0.05 is considered to be statistically significant. *p value<0.05, ***p value<0.001.



Graph 1. Distribution of mean candida colonies according to the histopathological findings (PAS) across three study groups



Graph 2. Distribution of site of lesion across two study groups

The distribution of habits differs significantly between oral cancer and control group as well as leukoplakia and control group (p<0.01). Out of 63 patients, 57 patient (90.5%) were found to be positive for candida colonies out of which 51 (89.5%) were having habits. Distribution of presence of candida colonies differ significantly according to habit the (p-value<0.05). In the present study, the most common site for leukoplakia and oral cancer was buccal mucosa the lesions defined in the study were mostly found in relation to buccal mucosa. We also found the distribution of mean candida colonies (colony forming units per ml) in oral cancer group were 613 ± 385.9. Out of 21 patients 5 (23.8%) patients were poorly differentiated, 10(47.7%) was moderately differentiated and 6 (28.6%) was well differentiated. In leukoplakia group 190 ± 116.7 and out of 21 patients in leukoplakia group 19 patients (90.5 %) had mild epithelial dysplasia, 2 patients (9.52 %) had moderate dysplasia.

Candida colonies were present in all the subgroups. In control group 79 ± 210.8. The distribution of mean candida colonies in oral cancer group was significantly higher compared to the leukoplakia and control groups (p-value <0.001 for both). **Table 3.** In oral cancer group, the mean of candida colonies present per ml of saliva in histopathologically Positive PAS was 656 ± 380.7 (cfu/ml) and with negative PAS 225 ±176.8 (cfu/ml). In leukoplakia group, the mean of candida colonies present per ml of saliva in histopathologically Positive PAS 300 ± 89.9(cfu/ml) and with negative PAS 122.7 ± 70.7 (cfu/ml) and in control, the mean of candida colonies present per ml of saliva in histopathologically Positive PAS 463 ± 428.5 (cfu/ml) and with negative PAS 15 ± 17.2 (cfu/ml). The distribution of mean candida colonies differs significantly between cases with negative and positive PAS in leukoplakia group and control group (p-value<0.001). Graph 1

DISCUSSION

In the oral carcinogenesis, several factors have been involved such as age, gender, ethnicity, lifestyle, genetic background, status of health and exposure to carcinogens. Since time immemorial, micro-organisms have been presumed to have an etiological role in the evolution of oral cancer. The present study evaluates the prevalence and correlation of candida with normal controls, leukoplakia and oral cancer patients. Our study included total 63 patients, 21 in each group as per inclusion criteria age ranging from 15 years to 74 years with Mean age found to be 43 years. Out of 63 patients 50 (58.2%) were male and 13(20.7%) were female. In the present study the mean age of the oral cancer group was 55.6 ± 9 with maximum of 74 and minimum of 40 yr of age. It was found that maximum patients were found to be above 55 years which is in concordance with Edwards BK *et al* (2002) [6,] Krishna A *et al* (2014)[7] . According to SEER (Surveillance Epidemiology and End Results) [8] fact sheet from 2005-2009, the median age of oral cancer was 62 years of age, whereas (Johnson 1991; Levi 1994)[9] and Ferlay *et al* (GLOBACON 2004)[10] reports were suggestive of increase in incidence among young adults (<45 years). Similarly the mean age among leukoplakia group was found to be 41.6 ± 13.6. It was found that maximum patients were above 41 years,similar findings were observed in Allen *et al* (2001)[11]. In present study out of 63 patients 22 (84.6%) patients who were in the age group of below 40 years showed candida colonies, 14 patients who were in age group of 40 -50 years were positive for candida colonies and 21 patients who are in the age group above 50 years all of them had candida colonies in saliva. Zaremba e*t al* (2006)[12,] Prakash B *et al* (2015)[13] also reported high incidence in middle age.

In our study Male predominance was seen in oral cancer and leukoplakia group, similar results were found by Krishna A *et al* (2014)[7,] Singh *et al* (2016)[14]. WHO (1984)[15] has estimated that 91% of oral cancer is directly attributable to tobacco usage. In our study we found smoking and smokeless form of tobacco and alcohol are known factors for causing oral cancer. All the 21 patients in oral cancer group and leukoplakia group were habitués. The distribution of habits differs significantly between oral cancer and control group as well as leukoplakia and control group ($p<0.01$)(Table 2) Similar findings were observed by Bhonsle *et al* (1979)[16], Jaber *et al* (1999)[17,] Riebel *et al* (2003)[18] and Napier *et al* (2008)[19] who stated that tobacco is a major independent risk factor for the development of cancers as well as oral pre cancers. Distribution of presence of candida colonies differ significantly according to habit (p-value<0.05) Candida colonization increases with tobacco habits as tobacco contribute to increase in the concentration of yeast by inducing changes in mucous membrane, releasing pro-candida factors found in tobacco and acidifying salivary ph, as favored by candida species. Similar results were found by Oliver *et al* (1984)[20] and Baboni *et al* (2009)[21] showed enhancement of candida histolytic enzymes and helps in adherence to mucosal surfaces. In the present study, the most common site for leukoplakia and oral cancer was buccal mucosa the lesions defined in the study were mostly found in relation to buccal mucosa.(Graph 2) Several studies report incidence of candidiasis being more commonly associated with respect to the same region. Whereas study conducted by A W Barrett *et al* (1998)[22] revealed total of 6.7% tongue biopsies shows fungal infection. In present study we found, the mean values of candida colonies (colony forming units per ml) in oral cancer group were 613 ± 385.9. Out of 21 patients 5 (23.8%) patients were poorly differentiated, 10(47.7%) was moderately differentiated and 6 (28.6%) was well differentiated. The distribution of prevalence of presence of candida colonies did not differ significantly across three subgroups of cancer ($p>0.05$), which is in concordance Sharma *et al* (2011)[2] and Samarnayake *et al*(1994)[23] who reported positive association between fungal infection oral neoplasia. In leukoplakia group the mean values of candida colonies (colony forming units per ml) was 190 ± 116.7 And Out of 21 patients in leukoplakia group 19 patients (90.5 %) had mild epithelial dysplasia, 2 patients (9.52 %) had moderate dysplasia.Candida colonies were present in both the subgroups. Prevalence of presence of candida colonies did not differ significantly between different grades of dysplasia (p-value>0.05) these results are in concordance with Sharma *et al* 9 (2011)[2] study (91%) and Dany A *et al* (2011)[24] (p value= 0.062) . Study conducted by Siddharth Singh *et al* (2014)[25] also showed no correlation between presence of candida and epithelial dysplasia. As per, McCullough M *et al* (2002)[26] the degree of epithelial dysplasia showed correlation with higher amounts of yeast in the oral cavity. In our study mean candida colonies in oral cancer group is significantly higher compared to leukoplakia group and control group ($p<0.001$ for both),(Graph 3) which is similar to study conducted by Nieminen *et al* (2009)[27], Bakri *et al* (2010)[28] Colonization of oral mucosa with Candida which is widely deemed to have the potential to produce carcinogenic amounts of ACH from both ethanol and glucose may play a considerably vital role in the development of oral cancer. The histopathological examination done showed the distribution of PAS differs significantly between oral cancer group, leukoplakia and normal control (p-value<0.001 for both), the results are in concordance with Kumar R S *et al* (2009)[29] with $P<0.001$. And Horstein *et al* (1979)[30] 71.5% oral cancer cases were positive for PAS.

The distribution of mean candida colonies differ significantly between cases with negative and positive PAS in leukoplakia group. The distribution of mean candida colonies differs significantly between cases with negative and positive PAS in control group. As the invasion and damage by candida depends on fungal morphology and activity, on the keratinocyte type and stage of differentiation, indicating epithelial cells differ in their susceptibility to the fungus. An effort was made in present study to find prevalence and correlation of candida with oral cancer and leukoplakia as in most of studies it has been concluded a positive role of candida in oral carcinogenesis by various mechanisms. So we found a positive correlation between candida colonies in saliva of oral cancer patient and leukoplakia. It is well known that candida species are commensals of oral cavity, but it is an opportunistic pathogen. This is very much evident in our study as and when the environment of oral cavity changes. More numbers of mean candida colonies were seen, i.e. seen with gradual increase in number of colonies from control group to leukoplakia and highest number noted in oral cancer group. Even the role of tobacco habituates also should be taken into consideration which by irritating the mucosal surface may create conducive environment to colonization of oral cavity. Though there are various factors coming into picture about how candida colonizes and nature of epithelial cells reacting to fungus and allowing penetration into deeper layers of the epithelium. So we conclude that there may be correlation of candida in causing dysplasia as well as has role in malignant transformation. We would propose to do more studies with more samples as the study is done in closed setting, cohort

studies need to be done to come to proper conclusion. In this study we have not taken in consideration the type of candida species and also if any other method of counting the colonies which will be precise and not vary in different studies so that standardization can be done. We would also like to propose studies to know the effect of antifungal therapy on colonization

Conclusion

The present study proposes that Candida may play a significant role in the development of oral cancer via its interaction with epithelial cells and formation of bioflim resulting in production of epithelial cytokines and MMPs and an epithelial pro-invasive phenotype. Further, this carcinogenic effect is likely linked with both the form of candida growth (whether yeast or hyphal)

Conclusions were drawn from the present study:

- Candida colonies and hyphae were detected in oral cancer and leukoplakia; the prevalence was a higher than healthy adults.
- On comparing the study groups the frequency of oral yeast carriage was significantly greater ($p < 0.05$) in the oral cancer than the leukoplakia and healthy controls.
- Quantification of candida carriage helps in determining an interaction between oral carriage of yeast and oral epithelial dysplasia.
- Saliva collection method is found to inexpensive, easy and reliable for quantitative measurement of candida colonies.

Although Candida species has been considered an etiologic factor for potentially malignant disorders, the pathogenesis is not clearly understood and is still a field under extensive research. Candida being a normal commensal in the oral cavity, its presence alone cannot be related to the etiology of leukoplakia and oral cancer. Thus, further research is needed to assess the invasive and carcinogenic nature of candida with keratinocytes of oral mucosa. It is anticipated that this study will enhance our understanding of the role of Candida in malignant transformation and oral carcinogenesis. Further extensive studies may advances our understanding and opens new mechanism in the prevention and treatment of oral cancer. The method of whole saliva collection is simple, practical, inexpensive and reliable, and it can be easily used by all dental practitioners. With the increasing numbers of geriatric and medically compromised patients being seen by dental practitioners, longitudinal evaluation of whole saliva should be considered as a part of clinical evaluation to identify patients at a risk for candidiasis and prevent clinical complications.

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