

# Identification of *Candida albicans* by using different culture medias and its association in potentially malignant and malignant lesions

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## Abstract

**Background and Objective:** The present study evaluates the association of *Candida albicans* with normal control group, potentially malignant and malignant lesions of oral cavity by using two different liquid culture media. **Materials and Methods:** Saliva was collected and biopsy was taken only from those clinically suspected potentially malignant and malignant lesions for histopathological diagnosis. Saliva samples were inoculated for fungal growth in Sabouraud's dextrose agar and culture-positive samples had undergone for Germ tube test. Germ tube-positive samples were further taken for quantification of chlamyospore production in liquid media at 8 and 16 hours. **Results:** In normal control groups no fungus growth was found; however, potentially malignant and malignant cases showed fungus growth, positive germ tube test and chlamyospore formation. The result also showed rapid and quantitatively more chlamyospore formation in corn meal broth + 5% milk in comparison to serum milk culture media. **Conclusion:** The oral mucosa is compromised in potentially malignant lesions, it can be argued that this species may be involved in carcinogenesis by elaborating the nitrosamine compounds which either act directly on oral mucosa or interact with other chemical carcinogens to activate specific proto-oncogenes and thereby initiate oral neoplasia.

**Keywords:** *Candida albicans*, chlamyospore, liquid media

## Introduction

Cancer afflicts all communities worldwide. Approximately 10 million people are diagnosed with cancer and more than 6 million die of the disease every year. Oral cavity cancer is currently the most frequent cause of cancer-related deaths among Indian men.<sup>[1]</sup> The strong association between cancers of the oral cavity and pharynx with tobacco use is well established. Alcohol use has been identified as a major risk factor for cancers of the upper aerodigestive tract. In India and Southeast Asia, the chronic use of betel quid in the mouth has been strongly associated with an increased risk for oral cancer. Recent evidence suggests that human papilloma virus (HPV) may be causative with some oral and oropharyngeal cancers. Dietary factors, such as a low intake of fruits and

vegetables, may also be related to an increased cancer risk.<sup>[2]</sup> Certain strains of *Candida albicans* and of other yeasts play a causal role in the development of oral cancer, by means of endogenous nitrosamine production.<sup>[3]</sup>

The oral cavity harbors hundreds of different microbial species and *C. albicans* is the most common fungal pathogen in humans. It 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.<sup>[4]</sup> In many states of impaired local and/or general health, *C. albicans* may assume a pathogenic role giving rise to acute and chronic clinical manifestations such as thrush, atrophic glossitis, leukoplakic lesions, angular stomatitis and others.<sup>[5]</sup> The presence of *Candida* in mouth together with epithelial changes may predispose to candidal infection which together with other cofactors may also induce epithelial dysplasia leading to malignant change. As the mucosa including epithelial physiology is likely to be distributed in oral submucous fibrosis, it is hypothesized that the oral yeast carriage in patient with this condition may be different from those who are healthy;<sup>[6]</sup> therefore the coexistence of *Candida* species within humans either as commensals or pathogens has been a subject of interest, among physicians. Also the association of *Candida* with various potentially malignant and malignant lesions has been reported as a causative agent.<sup>[7]</sup>

Hence the present study was undertaken to assess and identify the association of *C. albicans* in normal control group, potentially malignant and malignant lesions by using different liquid culture media.

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## Materials and Methods

The present study comprised of total 75 patients, which included, 30 potentially malignant (15- leukoplakia and 15- oral submucous fibrosis), 30 oral squamous cell carcinoma (OSCC) and normal controls group comprised of 15 healthy volunteers who were not having any relevant medical, dental and habit history.

All the subjects were asked to rinse the mouth with distilled water thoroughly to remove any food debris. After 10 min phosphate buffer solution was used as oral rinse method for saliva collection. Samples were obtained by requesting subjects to keep and swirl the solution for 1 min, and then expectorate all saliva into presterilized container without swallowing. After collection of saliva, biopsy was taken only from those who were clinically suspected as potentially malignant and malignant lesions for histopathological diagnosis.

### Preparation of culture media

#### For fungus growth

Sabouraud's dextrose agar- 65 g of the media was suspended in distilled water. It was mixed well until a uniform suspension was obtained. It was heated with frequent agitation and boiled and then sterilized at 118-121°C for 15 min. To prepare a selective culture medium aseptically Streptomycin was added for every milliliter of medium before use.

#### For germ tube test

Serum-human venous blood was collected and allowed to fully clot. The specimen was placed vertically in a test tube rack to speed up the clotting action. After clotting the specimen was placed in a centrifuge machine at 3000 rpm for 10 min.

#### For chlamyospore

Corn meal broth + 5% milk- corn meal agar media was modified to prepare the broth. It was mixed with cold distilled water, stirred at 6°C. Next day insoluble components were removed by filtration, then 5% pasteurized milk was added and autoclaved at 115°C for 30 min. It was allowed to cool and then poured into the test tubes.

Milk serum was prepared from nonpasteurized cow's milk. Sulfuric acid was added drop by drop in milk until it forms curd. The supernatant was removed and left over was filtered by filter paper until it became clear; then autoclaved at 115°C for 30 min. It was allowed to cool and then poured into the flask.

### Saliva culture technique for *Candida*

All samples were centrifuged at 1700 rpm for 10 min. Now the supernatant was discarded and sediment material was carried with pipette and inoculated in Sabouraud's Dextrose Agar (SDA) media. The sample was streaked using inoculating loop

and incubated in 37°C for 48 hours. The growth appeared in 48 hours as cream/white colored, smooth and pasty colonies.

### Identification of *Candida* species

#### Method

Very small inoculum from an isolated candidal colony was picked up with a sterile inoculating loop and was suspended in a test tube containing normal human serum (0.3-0.5 ml) by rubbing the inoculated loop against the wall of the test tube. This helps in diluting the pasty colonies by giving the serum turbid appearance. The mixture was incubated at 42°C for 2-3 hrs. A drop of mixture was placed in a clean glass slide and covered with a clean cover slip. This was first examined under a low-power objective to locate the group of cells and later, the presence of germ tube was confirmed under high-power objective of the microscope.

### Comparison of culture media for rapid and quantitatively more production of chlamyospore

Only the germ tube-positive samples were preceded for further study and to compare the rapid production of chlamyospore with two different culture media (corn milk broth + 5% milk and serum milk). Very small inoculums were picked up with a sterile inoculating loop and was suspended in same manner as it was done for Germ tube test. The two different test tubes containing corn meal broth + 5% milk and serum milk were placed in water bath at 45°C and results were read at 8 and 16 hours, respectively.

Chlamyospores were observed under wet mounts. A drop of inoculated broth media was placed onto the slide, over it a drop of lactophenol cotton blue stain (LPCB) was added then a cover slip was put over it. This was first examined under a low power objective to locate the group of cells and later, the presence of chlamyospores were confirmed and quantified in by two observers in 10 fields (high power view) in both media

### Histopathological diagnosis

In order to study the association of *C. albicans* and the types of mucosal lesions all the consecutive biopsies of oral mucosa were processed according to the standardized laboratory method. The histological sections were stained with Hematoxylin and Eosin for histopathological diagnosis.

## Results

In normal control groups no fungus growth was found, in potentially malignant eight cases showed fungus growth [Figure 1] out of 15 leukoplakia cases and three cases showed fungus growth out of 15 cases in oral submucous fibrosis cases where as in malignant (OSCC) 20 cases showed fungus growth out of 30 cases [Table 1]. All fungal growth positive cases further carried for germ tube test in serum media [Figure 2]. The result revealed that all leukoplakia and oral sub mucous fibrosis gave positive germ tube test but

in malignant, 14 cases showed positive germ tube test for *C. albicans* and six cases showed nonalbicans species out of total 20 cases [Table 2].

Further the confirmatory test for *C. albicans* were carried by using two different medias i.e., corn meal broth + 5% milk and serum milk for production of chlamyospore [Figures 3 and 4]. In 8 hours no growth was observed in potentially malignant lesions whereas chlamyospore formation occurred only four malignant cases in both media but in 16 hour all germ tube-positive potentially malignant cases showed chlamyospore formation and only 14 malignant cases showed chlamyospore formation [Table 3]. The quantification of chlamyospore was observed in 16 hours in both the lesions. Total chlamyospore formation in potentially malignant cases was highly significant ( $P < 0.001$ ) in corn meal broth + 5% milk in comparison to serum milk [Table 4]. In malignant cases total chlamyospore formation was also highly significant ( $P < 0.001$ ) in corn meal broth + 5% milk media in comparison to serum milk media [Table 5]. These results revealed that corn meal broth + 5%

milk media produced more number of chlamyospore in potentially malignant and malignant cases in comparison to serum milk media, but quantitatively more in malignant cases [Table 6].

This confirmatory test of chlamyospore showed that the association of *C. albicans* with potentially malignant and malignant were statically significant but highly significant ( $P < 0.001$ ) in malignant patients [Table 7] in comparison to normal controls.

## Discussion

Yeasts are commensal organisms found in approximately 40% of individuals, the predominantly species being *C. albicans*. It has the potential to infect virtually any tissue within the body, however, it's predominantly found on oral and vaginal mucosa.<sup>[8]</sup> Not all the people carry *C. albicans* and its colonization is not always continuous, the carriage rate varies from 1.0% to 80.6% depending upon the population surveyed.<sup>[9]</sup> There are possibility that yeast carriage changes in



**Figure 1:** Fungus growth in SDA media



**Figure 2:** Germ tube formation in human serum x40



**Figure 3:** Chlamyospore formation in corn meal broth + 5% milk with LPCB stain at 16 hours in malignant cases x40



**Figure 4:** Chlamyospore formation in serum milk with LPCB stain at 16 hours in malignant cases x40

**Table 1: Fungal growth with Sabouraud's agar media in normal control, potentially malignant and malignant cases**

	Normal control	Potentially malignant				Malignant	
		Leukoplakia	%	OSMF	%	OSCC	%
Positive growth	0	8	53.3	3	20	20	66
Negative growth	0	7	46.6	12	80	10	33
Total	15	15	100	15	100	30	100

**Table 2: Germ tube test with serum in potentially malignant and malignant cases**

	Leukoplakia	Potentially malignant		Malignant		
		%	OSMF	%	OSCC	%
<i>Candida albicans</i>	8	100	3	100	14	46
Non-albicans species	0	0	0	0	6	20
Total	8	100	3	100	20	100

**Table 3: Chlamyospore formation with serum milk in potentially malignant and malignant cases**

	Leukoplakia	Potentially malignant		Malignant		
		%	OSMF	%	OSCC	%
8 hours	0	0	0	0	0	0
16 hours	8	100	3	100	14	100

**Table 4: Total chlamyospore formation in potentially malignant (leukoplakia + OSMF) cases after 16 hours**

	No. of patients	Mean	SD	t	Df	Result
Serum milk	11	16.73	5.92	5.58	10	
Corn meal broth + 5% milk	11	41.36	16.32			***

**Table 5: Chlamyospore formation in malignant oral squamous cell carcinoma cases after 16 hours**

	No. of patients	Mean	SD	T	Df	Result
Serum milk	14	13.43	6.43	4.828	13	
Corn meal broth + 5% milk	14	39.00	18.37			***

**Table 6: Total chlamyospore formation with serum milk and corn meal broth + 5% milk in potentially malignant and malignant cases**

	Potentially malignant		Malignant		Total	
	N	%	N	%	N	%
Serum milk	184	28.79	188	25.61	372	27.09
Corn meal broth + 5% milk	455	71.21	546	74.39	1001	72.91
Total	639	100.00	734	100.00	1373	100.00

N- Total number of chlamyospore formation

**Table 7: Association of *Candida albicans* with normal controls, potentially malignant and malignant cases**

	Normal controls		Potentially malignant		Malignant	
	N	%	N	%	N	%
Positive cases	0	0.00	11	36.67	14	46.67
Negative cases	15	100.00	19	63.33	16	53.33
Total	15	100.00	30	100.00	30	100.00

 $\chi^2 = 10.05$ ; df = 2; Result:  $P < 0.01$  (highly significant)

frequency may be due to physiological changes related to age, body fluids, mucosal surfaces, natural barriers against yeast colonization, living environment, habits of the individual and changes to the ecological environment of the oral cavity.<sup>[10]</sup> Recently, an interest in the study of oral candidiasis has markedly increased mainly because of its association with viral infection due to human immunodeficiency, but also because of its relation with potentially malignant and malignant lesions of oral mucosa.<sup>[11]</sup>

The present study evaluated the association of *C. albicans* with normal controls, potentially malignant and malignant patients for which we used oral rinse method for saliva culture technique and took biopsy from the suspected lesion. However, the other authors suggested different sampling and identification methods for *Candida* spp. That would also certainly influence the results, because of the uneven distribution of *C. albicans* throughout the oral cavity; swab samples can yield false-negative culture more often than oral rinse samples or imprint culture.<sup>[10,12]</sup> Compared with the imprint method the rinse culture method has a markedly increased upper limit of detection in quantifying yeast carriage which is particularly useful when dealing with highly infested individuals.<sup>[13]</sup>

The association of *C. albicans* was significant with malignant cases in comparison to potentially malignant cases. In potentially malignant group the association of *C. albicans* with leukoplakia was more significant in comparison to oral sub mucous fibrosis lesions. The association of *C. albicans* with potentially malignant and malignant cases has been investigated by various authors under microbiological,<sup>[6,11,14-16]</sup> cytological<sup>[7]</sup> and histopathological studies.<sup>[6,7,11]</sup> Other authors has got slightly lower results (30%) candidal-positive culture in leukoplakia,<sup>[11]</sup> other precancerous lesions 48.88% and almost similar in cancer 60%.<sup>[7]</sup>

In present study supports that there is an association of yeast and its role in malignant transformation of leukoplakia, oral sub mucous fibrosis.

Other studies also supports that their may be an association of yeast and its role in malignant transformation of leukoplakia<sup>[3,11,17-19]</sup> oral sub mucous fibrosis<sup>[6]</sup> and OSCC.<sup>[8]</sup> It may well be that certain strains of *C. albicans* possess properties important in the development of pathological conditions and malignant changes.

*C. albicans* plays a causal role in the development of oral cancer, by means of endogenous nitrosamine,<sup>[3,9]</sup> oligosaccharide and lectin-like component production.<sup>[20]</sup>

In the present study saliva was inoculated in SDA media for fungal growth at 37°C - 2 days. Positive fungal growth cases were undergone for germ tube test to identify *C. albicans* at 42°C. We found two types of germ tubes. First, Germ tubes

were cylindrical outgrowths rose from blastospores and grown continuously by extension whereas second one is the filamentous outgrowths of *Candida tropicalis* which are known as pseudohyphae those were budding cells that remain attached to the blastospores and may elongate.

When pseudophyphae elongate, they may resemble germ tubes. Differentiation between the two depends on the presence of a constriction at the junction between the filamentous outgrowth and the mother blastospore in a pseudohypha and the absence of such a constriction in a germ tube.<sup>[21,22]</sup>

In our study confirmatory test of identification of *C. albicans* was carried out by formation of chlamyospore. Few authors have suggested that *Candida dubliniensis* is capable to produce germ tube as well as chlamyospores, its ability shared only with the closely related species *C. albicans* but the difference is that this species is unable to grow at 42-45°C<sup>[22]</sup> and is recovered primarily from the oral cavities of human immunodeficiency virus (HIV)-infected individuals and AIDS patients.<sup>[23,24]</sup>

In this study we compared two different liquid media for the rapid production of chlamyospores i.e., corn meal broth + 5% milk and serum milk. The quantification of chlamyospore was done at 8 and 16 hours. The result showed that the corn meal broth + 5% milk gave rapid and more numbers of chlamyospores in both lesions as compared to serum milk. Some authors have suggested that the time required to produce then with standard method is 48-72 hours in rice meal agar and tensoactive agents. This time can be reduced or sorted using liquid media such as corn meal broth and diary supplements.<sup>[11]</sup> Corn meal agar stimulates sporulation of *C. albicans*, and is useful in suppressing certain other fungal growth,<sup>[25]</sup> while milk enhances the formation of chlamyospore by 21.4-95.5%.<sup>[11]</sup>

In conclusion, as it is well documented in support of an association of *Candida* and its role in malignant transformation of leukoplakia, oral sub mucous fibrosis. This commensal appears to be the reservoir of infection, and when the optimal conditions supervene they may cause disease. As the oral mucosa is compromised in potentially malignant lesions, it can be argued that the presence of *Candida* species may involved in carcinogenesis by elaborating the nitrosamine compounds which either act directly on oral mucosa or interact with other chemical carcinogens to activate specific proto-oncogenes and thereby initiate oral neoplasia.

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