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Evaluation of *Candida* species in oral cavities of patients with oral submucous fibrosis and healthy controls: A comparative study

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ABSTRACT

Background: Oral submucous fibrosis (OSF) is one of the most abundant premalignant lesions in the oral cavity, in South Asian population (10.54%). Overgrowth of fungi, particularly *Candida* species, predisposes to the etiopathogenesis of premalignant lesions, developing them to oral malignancies. **Objective:** To quantitatively evaluate and determine the prevalence of opportunistic *Candida* species in OSF patients and healthy controls. **Methods:** Healthy controls (n = 28) and OSF patients (n = 28) with histopathologically confirmed diagnosis were recruited from the Oral Medicine Clinic of Dental Teaching Hospital Peradeniya. Samples were collected by oral rinsing technique, and they were inoculated on to Candida HiCrome agar for the isolation of *Candida* species. Colonies with different colors were counted followed by the germ tube test. The Mann-Whitney U test and Chi square tests were used for the statistical analysis. **Results:** OSF patients (78.60%) and only 53.6% of the controls were positive for *Candida* growth on culture. *Candida* was found to be present significantly higher ($p < 0.05$) in OSF group as compared to controls. *C. albicans* was the most predominant species in the cohort. In both study groups, *C. albicans*, *C. krusei* and C*. glabarata* were isolated. **Conclusion:** This study depicted a significantly higher *Candida* growth in OSF patients than the controls, with *C.albicans* being the predominant species. This suggests that, OSF-mediated mucosal changes facilitate *Candida* colonization in the oral epithelium.

Keywords: Candida growth, oral submucous fibrosis patients, controls, oral cavities, malignant transformation

INTRODUCTION

Oral Submucous Fibrosis (OSF) is a chronic, progressive, and potentially malignant condition. It affects the oral mucosa, leading to fibrosis, scarring, and the gradual loss of mouth mobility (1). This condition is closely related to betel nut chewing, tobacco usage, and a variety of other environmental and genetic factors (2). Although *Candida* is deemed a normal flora comprising 3-47% of the oral cavity in healthy people, it causes common fungal infections in the oral cavity when our immune system is disturbed (3). Among the microbial agents in the oral cavity which are involved in the pathogenesis of OSF, *Candida* spp. is recognized as a contributing factor due to its

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ability to colonize the oral cavity in compromised mucosal environments. Several studies have found that the prevalence of *C. albicans* is high in OSF patients (4). Mucosal histopathological alterations in OSF such as fibrosis, impaired mouth opening and poor oral hygiene, toughness, impaired local immunity pave the way to a favorable environment for the overgrowth of *Candida* in OSF patients. In addition, the overgrowth of *Candida* plays a role in the pathogenesis of stomatopyrosis in patients, which is the most common symptom in patients with OSF for them to consult a doctor (5). Overgrowth of *Candida* in the oral cavity promotes the transformation of pre-malignant lesions such as OSF into oral carcinoma either by producing carcinogenic nitrosamines or by converting pro-carcinogens into more virulent carcinogenic compounds. *Candida* overgrowth leads to chronic inflammation in the subjacent oral mucosa, producing cytokines and chemokines by the activated immune cells during chronic inflammation. This stimulates tumor growth and progression ending up with the transformation of oral malignancy (6).

Candida albicans is the most predominant species, being the most virulent pathogen possessing the most carcinogenicity out of the other *Candida* species (7).

Yet, few studies have reported an escalated prevalence of isolation of other non-*Candida albicans* species for instance, *Candida glabrata*, *Candida tropicalis*, *Candida krusei, Candida parapsilosis* and *Candida dubliniensis* since past 20 years, owing to different factors like immune-suppressants and prolonged use of broad-spectrum antibiotics and antifungal drugs (8), (9), (10). Hence, the identification of candidal carriages and the most abundant species in the oral cavities of OSF patients is of paramount importance in helping the overall improvement of the prognosis of the disease preventing the transformation into oral carcinoma since the application of therapeutic agents depends on the abundant *Candida* strain in the oral cavity (9).

Objectives

The aim of this study was to quantitatively assess the growth of different *Candida* species between OSF patients and controls and its association with risky habits in the Sri Lankan population.

METHODOLOGY

This was a case control study, comprising 28 participants per each study group; test group and the control group. All together 56 participants were recruited by convenient sampling technique. Ethical clearance was obtained by the ethics review committee of the Faculty of Allied Health Sciences, University of Peradeniya. (AHS/ERC/2022/064).

Study participants, who had given written consent to participate in the study, were recruited from the Oral Medicine Clinic of Dental Teaching Hospital, Peradeniya from June 2022 to September 2022. The eligibility criteria for the test group were, above 18 years of age, participants who had a clinically & histopathologically confirmed diagnosis of OSF, who had no other chronic systemic diseases such as cancer, chronic kidney disease, diabetes, etc., and who had not taken antibiotics, antifungal drugs and, corticosteroids within the last six months. Age and sex-matched participants who did not have a clinically & histopathologically confirmed diagnosis of OSF, the participants who had not taken antibiotics, antifungal drugs, and corticosteroids within the last six months, and who had no systemic diseases were included in the control group. The control group was recruited from the Oral Medicine Clinic of Dental Teaching Hospital Peradeniya and from the Dental Faculty staff. Data on socio-demographic details, medical history, behavioral patterns such as tobacco use, betel quid chewing, and alcohol usage were collected using an interviewer administered questionnaire. Data collection on socio-demographic and behavioral data was done using an interviewer-administered questionnaire.

The donors were asked to rinse the mouth with sterile normal saline and spit the oral content into a sterile falcon tube. Laboratory work was conducted at the Microbiology Laboratory of Faculty of Dental Sciences and the Faculty of Allied Health Sciences, University of Peradeniya. The collected samples were kept in the refrigerator at 4 ^{0}C for further processing. Samples were

centrifuged at 2500 rpm for 10 minutes, and the pellets were dissolved in 1 ml of sterile phosphate buffered saline (PBS).

Standards of different *Candida* species were first inoculated onto the Candida HiCrome agar plate as positive controls and sterile PBS was also inoculated as the negative control.

Approximately 25 µl of the dissolved pellet was inoculated on Candida HiCrome agar and incubated at 37 ^{0}C for 48 hours [3]. The presence of colonies with different colors on the Candida HiCrome agar plate was observed after the proper incubation. The germ tube test was done to the culture positives to confirm the isolation of *C. albicans* and colony counting was done to all the colonies and reported as the number of colony-forming units (CFU) per 1 ml of the sample. Statistical analysis was done using Excel and IBM SPSS Statistics version 22. Descriptive data was presented as medians with IQR and as percentages. Inferential statistics were done using non-parametric tests. The associations between the categorical variables were assessed by Chi-Square test of Independence, and Fisher's Exact Test. The associations between the *Candida* colony count and categorical variables were analyzed using Kruskal Wallis Test and Mann Whitney U test. A 95% confidence interval (CI) was applied and p -value less than 0.05 was considered statistically significant.

Ethical clearance was obtained by the ethics review committee of the Faculty of Allied Health Sciences, University of Peradeniya. (AHS/ERC/2022/064).

RESULTS

A total number of 56 individuals were examined where the test group consisted of 28 OSF patients. The control group (n=28) included age and sex-matched healthy individuals. The mean age of the test group was 47 (± 15.4) years, while it was 45 (± 11.3) years in the control group. Approximately, 82.1% (n = 23) were males in both study groups while 17.9% (n = 5) were females. The majority of the study group had up to secondary education (78.6%, n = 22 in both groups) (Table. 1). There was no significant association of the educational levels of the participants with the prevalence of OSF ($p > 0.05$).

Table 1: Socio-demographic details of the study cohort

Characteristic	OSF	Control	p value
Sample size	28	28	
Age (years)			
Mean	47	45	
SD	15.7	11.3	
Gender			1.000 $^{\chi}$
Male	82.1% (n = 23)	82.1% (n = 23)	
Female	17.9% (n = 5)	17.9% (n = 5)	
Education			0.377 ^{F}
Primary school education	14.3%	3.6%	
Secondary school education	78.6%	78.6%	
Diploma or certificate course	7.1%	14.3%	
Degree or postgraduate	0%	3.6%	

$^{\chi}$p value from Chi Square test
^{F}p value from Fisher's Exact Test

The percentages of current risk behaviors are shown in the Table 2. Approximately, 57.1% (n = 16) of the OSF patients were current tobacco users, while it was only 35.7% (n=10) in the control group. The Chi-Square test was performed to evaluate the association of tobacco exposure between the test group and the control group, The Person Chi-Squared test depicted a statistically significant association between the tobacco exposure and the presence of OSF at 5% significance level, χ^2 (1, N = 56) = 4.595, ($p < 0.05$).

In addition, using smokeless tobacco particularly associated with the presence of OSF.

Table 2: Comparison of current behavioral factors among the test group and the control group

Habit	Test group N (%)	Control group N (%)	p value
Tobacco users			**< 0.05$^{\chi}$**
Yes	16 (57.1)	10 (35.7)	
No	12 (42.9)	18 (64.3)	
Smoking tobacco	10 (35.7)	8 (28.60)	>0.05$^{\chi}$
Beedi	1 (3.60)	0 (0.00)	
Cigarette	5 (17.9)	5 (17.9)	
Both	4 (14.3)	3 (10.7)	
Smokeless tobacco	15 (53.6)	2 (7.10)	**< 0.05^{F}**
Tobacco powder	6 (21.4)	1 (3.60)	
Mawa	4 (14.3)	0 (0.00)	
Pan masala	2 (7.10)	1 (3.60)	
Khaini	2 (7.10)	0 (0.00)	
Babul beedi	1 (3.60)	0 (0.00)	
Betel quid			>0.05$^{\chi}$
Yes	13 (46.4)	10 (35.7)	
No	15 (53.6)	18 (64.3)	
Alcohol			>0.05$^{\chi}$
Yes	11 (39.3)	14 (50.0)	
No	17 (60.7)	14 (50.0)	
Areca nut			>0.05$^{\chi}$
Yes	13 (46.4)	10 (35.7)	
No	15 (53.6)	18 (64.3)	

$^{\chi}$-p value from Chi-Squared Test of Independence
^{F}p value from Fisher's Exact Test

The median *Candida* colony count in the cohort was 120 CFU/ ml (IQR 0 - 3930 CFU/ml). Among OSF patients, 32.1% (n = 9) had individual isolation of *C. albicans* while only 3.60% (n = 1) had individual isolation of *C. krusei*. Apart from the individual isolation, 42.9% (n = 12) of patients had simultaneous isolation of *C. albicans* + *C.krusei.* In addition, 7.14% (n = 2) of patients had simultaneous isolation of *C. albicans* + *C.glabarata* (Graph.1).

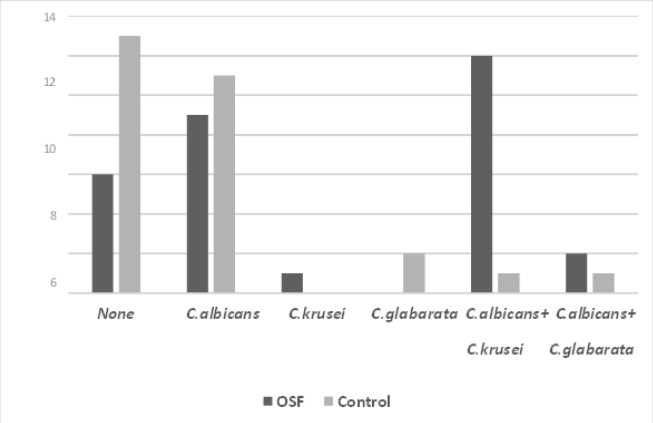

Figure 1: Distribution of *Candida* species in OSF patients and controls

In the cohort, OSF patients had a statistically significant higher CFU/ml of *Candida* than the controls (p = 0.006) (Table 3.).

Table 3: Concentration of *Candida* in OSF and control groups

Study group	Median CFU/ml	IQR	Range	p value
OSF	1860	8666	56200	**0.006**[M]
Control	40	330	24000	

[M] p value from Mann- Whitney U test

DISCUSSION

This study focused on the relationship between *Candida* growth in patients with OSF compared to controls. The most significant results are discussed below in detail.

In this study, *Candida* colony counts in OSF patients as compared to controls, presented a statistically significant higher growth. The median colony count, was significantly higher in OSF patients (1860 CFU/ml) compared to the controls (40 CFU/ml), indicating a greater susceptibility of OSF patients to *Candida* colonization. Although OSF arises in the submucosa of the oral cavity, this may progress to the epithelium developing reduced vascularity, epithelial atrophy, and, decreased mouth opening, which are the cardinal signs of OSF. These consequences might impair the mucosal immunity and epithelial barrier resulting, an alteration in the oral microenvironment facilitating *Candida* colonization in the epithelium. This *Candida* colonization is not only a marker of impaired oral immunity, but can predispose the epithelial tissue into malignant transformation by nitrosation and producing acetaldehydes, particularly by *C. albicans* (11, 12). These carcinogenic metabolites interact with epithelial cells predisposing the development of oral malignancies in the epithelium.

Our findings are thus in agreement with previous studies, such as B. George (13), who also found increased *Candida albicans* colonization in OSF patients. Moreover, this study reported simultaneous isolation of *C. krusei*, and *C. glabarata* with *C. albicans* in both controls and OSF patients.

Anila, et al also reported a similar result of a co-isolation of *C. kruei* and *C. tropicalis* with *C. albicans* in OSF patients in India (3). Majority of the people have one strain of *Candida* at one body site. However, if a person is immune compromised, he might harbor more than strain of *Candida* in his oral cavity (18).

This study found that OSF patients had a statistically significant higher *Candida* growth than the controls.

Yet, an Indian study conducted by Anila et al concluded that, although *Candida* growth is higher in OSF patients than the controls, it is insignificant, which might be due to the geographical and behavioral differences in Sri Lankan and Indian populations (3).

Another Sri Lankan study conducted in Peradeniya in 2006 (4), reported that the *Candida* carriage was insignificantly higher in OSF patients than the controls, which is contradictory to the present finding in the same geographical region. This could be due to the differences in the habits of the study participants since there is a huge time gap between 2006 and 2022. Tobacco usage was very high among OSF patients in this cohort (57.1%). However, there was no significantly higher *Candida* growth in tobacco users than in non-tobacco users in the OSF group.

On the other hand, in this cohort, *Candid*a growth was higher among tobacco users than in non-tobacco users in the control group, which is in congruence with the B. George's study, where they found a significantly high *C. albicans* growth in controls exposed to tobacco use (13). This observation can be supported by the findings of Darwazeh et al who also reported a significant increase of *Candida* colonization in healthy smokers (14). This indicates that tobacco may enhance *Candida* growth in healthy individuals. Tobacco smoking increases oxidative stress, and proinflammatory processes while reducing the immune mechanisms in the oral cavity by reducing cytokines, polymorphonuclear cells, and other enzymes which eventually lead to the impairments of both cellular and humoral immunity (15).

Tobacco consumption, either smoking or smokeless tobacco, increases *Candida* colonization, owing to reduced salivary immunoglobulin A, increased epithelial keratinization, and dysfunctional leukocytes (16, 17). However, the effects of tobacco may be masked by the pathophysiology of the disease, in the case of OSF patients in this study.

These findings strengthen the need for more targeted research into tobacco impact. In both OSF patients and controls in the present study, there was no significant difference in *Candida* CFU/ml between users and non-users of betel quid, areca nut, or alcohol. These findings suggest that these habits may not independently have an impact on the growth of *Candida*; however, in synergy with other risk factors such as tobacco or systemic immunosuppression, this might play a role.

This study found no gender-based differences in *Candida* CFU/ml in either OSF patients or controls. This is consistent with other studies showing that oral *Candida* colonization was not affected by gender (3, 11).

Although this is a very informative study, various variables might have impacted on the results. More accuracy in *Candida* detection may be achieved by combining culture-based techniques with molecular identification techniques like PCR or biochemical tests such as yeast assimilation test, and chlamydospore test for further identification (12). Utilization of automated microbial identification systems such as Auto Microbic Vitek system (Vitek 60, Bio- Merieux, France) is also deemed an effective way of identification of *Candida* strains (14). Such considerations point to areas of refinement in future studies.

CONCLUSION

This study depicted a significant increase in *Candida* colonization in OSF patients relative to healthy controls, indicating greater susceptibility to *Candida* colonization in individuals with OSF.

The higher frequency of co-isolated species like *Candida glabrata*, *Candida krusei*, and *Candida albicans* in OSF patients, as well as the most abundant prevalence of *Candida albicans*, raise the possibility of immunosuppression or changes in the oral microenvironment linked to OSF. Risky behaviors like tobacco use linked to increased *Candida* growth in healthy people.

These results highlight the necessity of more research on the association of prevalence of OSF and *Candida* growth, particularly to assess how OSF-mediated mucosal changes facilitate *Candida* colonization and how systemic and oral risk factors might interact in this process.

Author declaration

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Authors' contributions:
Conducting experiments, collecting data, analysis, manuscript drafting: PDMKCP; Conceptualization and planning experiments: RDJ; Conceptualization, planning experiments and providing statistical support and revision: NSP

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Data will be available on request from the corresponding author.

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