

Prevalence of *Candida* Carriage and *in Vitro* Evaluation of Phospholipase and Haemolysin Activity of Oral *Candida albicans* among Tobacco Users and Smokers in Dharan, Nepal

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ABSTRACT

Objectives: The main objective of this study was to determine the prevalence of *Candida* carriage among tobacco users and smokers along with *in vitro* evaluation of phospholipase and hemolysin activity of *Candida albicans*.

Methods: A laboratory based cross-sectional study was carried out in Dharan Sub-Metropolitan city, Eastern Nepal from June 2018 to November 2018. During the study 150 oral rinse samples were obtained from smokers (50), smokeless tobacco consumers (50) and non-tobacco users (50) as control group. The participants were provided 10 ml of normal saline and were asked to oral rinse for 1 minute. Oral rinse was collected in a sterile screw capped container and was transported to microbiology laboratory by maintaining the cold chain. The oral rinse sample was inoculated onto the Sabouraud dextrose agar with chloramphenicol and was incubated at 37°C for 3-4 days. The number of colonies of *Candida* was counted and *C. albicans* were identified by cultural characteristics, staining, germ tube test and chlamydospore formation test.

Results: The prevalence of *Candida* carriage was reported to be 22 (44%) in smoker group, 26 (52%) among smokeless tobacco users and 13 (26%) among control groups. The prevalence of *Candida* carriage was found to be significantly higher in the study group associated with tobacco chewers (P=0.008). However, the *Candida* carriage among smoker's group was not found to be statistically significant (P=0.059). Isolation of *Candida albicans* was higher among smokeless tobacco users 15 (30%), smokers 5 (10%) and non-users 6 (12%).

Conclusion: Colonization and carriage of *Candida* in the oral cavity of smokers and tobacco chewers were found to be higher than in controls. In addition, individuals with poor oral hygiene increase the risk of *Candida* colonization and infection under host debilitated condition.

Key words: *Candida*, Smokers, tobacco chewers, oral candidiasis, phospholipase, hemolysin

INTRODUCTION

Candida are almost universal on normal adult skin and *Candida albicans* is part of the normal flora of the mucous membranes of the respiratory, gastrointestinal, and female

genital tracts (Spampinato and Leonardi, 2013). *C. albicans* lives in oral cavity of 40% of the healthy human population with no harmful effects (Jenkinson and Douglas, 2002).

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However, host becomes susceptible to infection in debilitated or immunocompromised condition (Lopez-Martínez 2010). In oral candidiasis the most commonly identified pathogen is *C. albicans* (Manfredi et al. 2004). *C. albicans* pathogenesis is described by its host defense mechanism, adherence, and production of tissue degrading hydrolytic enzymes like protease, phospholipase and haemolysin (Pandey et al. 2018).

The secretion of hemolysin is followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis (Tsang et al. 2007). Phospholipase enzyme digests the phospholipid in the host cell membrane, producing cell lysis and changes in the surface characteristics that aid adhesion and infection. As a result, phospholipase production might be utilized to discriminate virulent invasive strains from non-invasive colonizers (Sachin et al. 2012). Therefore, phospholipase and hemolysin tests are important to identify and study the pathogenic strains of *C. albicans* in respect to the invitro activity of their hydrolytic enzymes.

Oropharyngeal candidiasis is an infection in the mouth and throat area. Usually, it is characterized by the formation of white patches on top of the tongue and throughout the mouth, which is also known as 'thrush' (Patil et al. 2015). Clinically, oral candidiasis may present as pseudomembranous candidiasis, erythematous candidiasis, hyperplastic candidiasis, denture-associated erythematous candidiasis, angular cheilitis, median rhomboid glossitis and chronic mucocutaneous candidiasis (Napenas et al. 2009; Farah et al. 2010). Pseudomembranous candidiasis or thrush is the most common presentation of oral candidiasis (Akpan and Morgan, 2002). It presents clinically as confluent whitish-yellow creamy or yellow velvety plaques on the surfaces of the oral mucosa (Reichart et al. 2000; Farah et al. 2010).

Candida has known to be opportunistic pathogen under tobacco chewing conditions (Javed et al. 2014; Hsia et al. 1981). It is suggested that individuals chewing tobacco are susceptible to oral *Candida* infections than non-chewers (Abduljabbar et al. 2017). Clinically, there are some factors that predispose to oral candidiasis including drug therapy, especially broad-spectrum antibiotics, immuno modulatory and xerogenic medications, blood dyscrasias and malignancy, dietary factors, endocrine disorders, immunologic disorders, and salivary changes (Ashman and Farah, 2005). Local factors that predispose to oral candidiasis include irritation from ill-fitting dentures and poor oral hygiene (Ashman and Farah, 2005).

Studies have shown that the innate and adaptive immune systems play a role in the clearing of fungal growth. T Helper

1 cells are known to produce cytokines that activate phagocytes to clear the pathogens (Romani 2000). There is a significant burden of serious oral and oropharyngeal infections in Nepal (Khwakhali and Denning, 2015). In immunocompromised people, oropharyngeal candidiasis is a prevalent opportunistic infection (Lamichhane et al. 2020). To best of my knowledge, this is the first study on assessment of oral *Candida* carriage among smokers and tobacco users from Dharan. Lack of epidemiological estimates can increase the risk of infections among susceptible groups of community. So, it was essence to evaluate the health of smokers and tobacco chewers in perspective to oral pathology. Therefore, this study was conducted to determine the prevalence of *Candida* carriage from tobacco users and smokers in Dharan, Nepal. In addition, this study aimed to characterize *C. albicans* on basis of Phospholipase and hemolysin activity.

METHODS

Study Design

This was the cross-sectional laboratory based study conducted from June 2018 to November 2018 in which 50 smokers, 50 smokeless tobacco consumers and 50 non-tobacco consumers (control) were included. During the study 150 oral rinse samples were analyzed in Microbiology laboratory of Central Campus of Technology, TU, Hattisar, Dharan. This research was conducted after receiving Ethical approval from Nepal Health Research Council (NHRC approval Registration no. 296/2018), Kathmandu, Nepal. Informed consent and socio-demographic information from participants were obtained through written form and questionnaire respectively.

Inclusion and Exclusion criteria

Inclusive criteria included active smokers, smokeless tobacco (*Paan and Gutka*) consumers and non-tobacco users as control group. Exclusion criteria included people with alcohol intake, on antibiotics, antifungals and steroids treatment for last 3 months, having a partial or complete dental prosthesis, and having diseases such as oral candidiasis, cancer, organ transplant patient, diabetes mellitus, hepatitis B and hepatitis C infections, HIV infection.

Sample collection

The participants were allowed to rinse 10 ml of sterile saline for 1 minute and oral rinse was collected by spitting in a broader capped sterile container. The oral rinse samples were transported to microbiology laboratory, maintaining cold chain on same day. All the collected samples were labeled with participant's identification number and processed within 2 hours of collection. In case of delay, the sample was usually stored at 4°C in the refrigerator.

Isolation and identification

A 50 µl of oral rinse sample was inoculated in Sabouraud dextrose agar (HiMedia, India) with chloramphenicol (0.05 gm/l) and incubated at 37°C for 3-4 days. Pure culture was identified by colony characteristics and simple staining. The number of colony was counted by colony counter and expressed as CFU/ml. For the identification of *C. albicans*, germ tube and Chlamydospore formation was evaluated as described by Deorukhkar and Roushani, (2018). For germ tube test the pure isolated colony of *C. albicans* was dispensed in 0.5 ml of serum and incubated at 37°C for 2 hours. After incubation the aliquot was taken in a clean slide and was observed under oil immersion for the formation of germ tube. In addition, Chlamydospore formation test was performed in which the pure isolated and suspected colony was cultured in corn meal agar (HiMedia, India). *Candida* that could form germ tube and Chlamydospore in corn agar was identified as *C. albicans*.

Phospholipase assay

The phospholipase test was done according to Samaranayake et al. (1984). *C. albicans* growth suspension was maintained at 0.5 McFarland standards. Egg yolk agar media was inoculated by 10 µL of the inoculum and allowed to dry at room temperature. The plates were incubated at 37°C for 48 hours. Phospholipase index (Pz) was measured by dividing the diameter of the colony by the sum of diameter of the colony and the zone.

Hemolysin assay

Hemolysin activity was evaluated according to Tsang et al. (2007). Hemolysin production by *C. albicans* was performed by inoculation overnight culture of yeast on sugar-enriched sheep blood agar. The blood base agar media was prepared by adding 5-7 ml of fresh blood to 100 ml Sabouraud glucose agar with 3% glucose. *C. albicans* growth suspension was maintained at 0.5 McFarland standards. About 10 µL of the yeast inoculum was placed at the center of the plates. The plates were incubated at 37°C in 5% CO₂ for 48 hours. Hemolytic Index (Hz value) was calculated by dividing the total diameter of the colony by the translucent halo zone.

Quality Control

Strains of *Candida albicans* ATCC 24433 was used as reference strain for the study. Reagents and culture media were regularly checked for their expiry date and performance. Equipment was standardized, optimized and its performance was checked through positive and negative controls.

Data analysis

The data was documented in MS-EXCEL 2010 was analyzed using statistical Package for Social Sciences (SPSS) version

16.0. Chisquare (χ^2) was used for statistical analyses. The p value of equal or less than 0.05 at 95% confidence interval was used for statistical significance.

RESULTS

The prevalence of *Candida* carriage was reported to be 22 (44%) in smoker group, 26 (52%) among smokeless tobacco users and 13 (26%) among control groups. The prevalence of *Candida* carriage among tobacco chewers and non-chewers was found to be statistically significant (P= 0.008). However, the carriage of *Candida* among smokers and non-smoker was not found to be statistically significant in this study (P= 0.059). The incidence of *Candida* carriage among smokers and smokeless tobacco users were similar. Isolation of *Candida albicans* was higher among smokeless tobacco users 15 (30%), smokers 5 (10%) and control 6 (12%) (Table 1).

The highest colony forming unit of *Candida* carriage was reported among smokers (220-1350 CFU/ml) and smokeless tobacco users (456-1900 CFU/ml) than in control groups (124-800 CFU/ml) (Table 2).

The phospholipase activity was screened from 13 (26%) of *C. albicans* isolated from smokeless tobacco users with Pz values range of 0.65-0.77 and 11 (22%) *Candida albicans* were screened for haemolysin activity with Hz range from 0.64-0.85. The phospholipase activity was screened from 5 (10%) of *C. albicans* isolated from smokers with Pz values range of 0.66-0.68 and 4 (8%) *C. albicans* were screened for haemolysin activity with Hz range from 0.63-0.84. The phospholipase activity was screened from 4 (8%) of *C. albicans* isolated from control groups with Pz values range of 0.62-0.73 and 2 (4%) of *C. albicans* were screened for haemolysin activity with Hz range from 0.62-0.82 (Table 3). *C. albicans* isolates from smokeless tobacco users and smokers were found to express high degree of phospholipase and hemolytic activity compared to control.

Status of Oral Hygiene

In this study, 38 (76%) of smokers were reported to brush their teeth once a day, 5 (10%) of smokers were reported to brush their teeth twice a day, 5 (10%) smokers were reported to brush their teeth sometimes and rest 2 (4%) never brushed their teeth. Similarly, 28 (56%) of smokeless tobacco consumers were reported to brush their teeth once a day, 7 (14%) were reported to brush their teeth twice a day, 10 (20%) were reported to brush their teeth sometimes whereas 5 (10%) never brush their teeth. In case of control group, 28 (56%) were reported to brush their teeth once a day and rest 22 (44%) were reported to brush their teeth twice a day. The prevalence of *Candida* carriage among groups with good and poor oral hygiene was statistically significant (P=0.001).

Table 1: Prevalence of *Candida* carriage and *Candida albicans* in study groups.

Study Population (n)	<i>Candida</i> Carriage N (%)	<i>Candida albicans</i> N (%)
Smokeless tobacco consumer (50)	26 (52%)	15 (30%)
Smokers (50)	22 (44%)	5 (10%)
Control groups (50)	13 (26%)	6 (12%)

Table 2: CFU/ml of *Candida* carriage among study groups.

Study Population (n)	Minimum CFU/mL	Maximum CFU/mL	Mean	Standard deviation
Smokeless tobacco users (50)	456	1900	980.30	365.03
Smokers (50)	220	1350	814	353.83
Control groups (50)	124	800	460.76	228.41

Table 3: Phospholipase (Pz) and Haemolysin (Hz) Index of isolated *Candida albicans*.

Study population	No. of Phospholipase producing <i>C. albicans</i>	Pz Range	No. of Hemolysis producing <i>C. albicans</i>	Hz Range
Smokeless tobacco users (50)	13 (26%)	0.65-0.77	11 (22%)	0.64-0.85
Smokers (50)	5 (10%)	0.66-0.68	4 (8%)	0.63-0.84
Control groups (50)	4 (8%)	0.62-0.73	2 (4%)	0.62-0.82

DISCUSSION

Candida being a part of normal flora of oral cavity can cause opportunistic infections when host is compromised (Jayachandran et al. 2016). Oral candidiasis is associated with poor oral hygiene, diabetic conditions, immunosuppressive therapy in cancer disease, and intake of tobacco (Guggenheimer et al. 2000).

Chewing tobacco that includes *Gutka*, *Betel quid* (BQ) which is common habit in South Asian nations like in India, Pakistan, Bangladesh, Sri Lanka and Nepal. *Betel quid* is mixture of areca-nut, lime enveloped in piper betel leaf whereas *Gutka* is found in sachet (Javed et al. 2013). The possible explanation for greater *Candida* colonization in *Gutka* (smokeless tobacco) consumers could be due to the presence of nicotine and hydrocarbons such as polycyclic aromatic hydrocarbons acting as nutrient for oral yeast facilitating its growth (Abduljabbar et al. 2017; Hsia et al. 1981). Tobacco usage leads to an increase in thickness of epithelial keratinized layer (Bancozy et al. 2001), decrease in levels of salivary IgA and suppression in functions of polymorphonuclear leukocytes (Bennet and Reade 1982, Keten et al. 2015), thus facilitates the proliferation of *Candida* species. *Candida* has known to be opportunistic

pathogen under immunosuppression and tobacco chewing conditions (Javed et al. 2014; Hsia et al. 1981).

In consistent to other studies even in this study, higher prevalence of *Candida* carriage was found in smokeless tobacco chewers. The prevalence of *Candida* carriage was reported to be 26 (52%) among smokeless tobacco users and 13 (26%) among control groups. The *Candida* colonization and tobacco chewing was statistically significant (P=0.008). However, Javed et al. (2014) showed no significant difference in oral *Candida* colonization among tobacco chewers and non-chewers (control). This contradiction in the study could be due to difference in sample collection techniques involved in complete enumeration of *Candida* from oral cavity.

In this study the *Candida* carriage among smokers was higher in comparison to non-smokers (controls). The prevalence of *Candida* carriage was reported to be 22 (44%) in smoker group and 13 (26%) among control groups.

Keten et al. (2015) reported highest oral *Candida* colonization among smokers and smokeless tobacco users. It is also hypothesized that cigarette smoke enhances adhesion,

growth and biofilm formation of *C. albicans* (Semlai et al. 2014). This study is consistent with the literature. In this study, no significant differences were observed between *Candida* carriage with age, gender, number and duration of smoking, duration of smokeless tobacco usage.

The phospholipase and haemolysin assay of isolated *C. albicans* from smokeless tobacco users and smokers were found to be higher in comparison to control groups. Hydrolytic enzymes confer microbial pathogenicity by mediating its adhesion, tissue damage, immune system evasion and dissemination. The Pz index was higher among test subjects than in controls which agreed to study by Maheronnaghsh et al. (2019).

In this study Hz index of oral *C. albicans* was higher among smokers and smokeless tobacco users. Hemolysin is another virulence factor that degrades the red blood cells of host and iron is released which is taken up by the yeast cells (Pandey et al. 2018). Tsang et al. (2007) reported all *C. albicans* isolates from oral cavity positive for hemolysin activity. The reason and mechanism behind increased phospholipase and hemolysin activity of *C. albicans* isolated from Smokers and smokeless tobacco users cannot be explained from this study.

Poor oral hygiene in smokers and smokeless tobacco users may contribute to higher oral *Candida* carriage rates in this study. The prevalence of oral *Candida* carriage in healthy control groups having good oral hygiene was low as compared to others. The lower frequency of *Candida* carriage in healthy control might be due to maintenance good oral hygiene and practice. This study can explain that being a part of normal flora the colonization and proliferation of *Candida* is indeed required before establishing infection.

CONCLUSION

This study concludes that prevalence of oral *Candida* carriage was significantly higher in smokers and smokeless tobacco users compared to non-users. Higher prevalence of *Candida* in poor oral condition alarms oral health warnings. *Candida* induces infection under immune-compromised and host predisposing conditions, the poor oral hygiene risks the host to oral candidiasis. Thus, strong preventive measures to abstain from tobacco products can reduce the risk of oral infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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