ANTIFUNGAL SUSCEPTIBILITY OF BIOFILM PRODUCING *Candida albicans* ISOLATED FROM ORAL CAVITY OF DIABETIC AND NON-DIABETIC POPULATION OF DHARAN, NEPAL

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Abstract

Background: *Candida* are almost universal on normal adult skin and *C. albicans* is part of the normal flora of the mucous membranes of the respiratory, gastrointestinal, and female genital tracts. It is acknowledged that diabetic patients are more susceptible to infections caused by *Candida albicans* due to increased blood glucose and inability of immune system in eradicating the fungus. Studies suggest that Gutkha consumers are also at high risk of oral *Candida* carriage. Materials and methodology 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 in cold chain to microbiology laboratory. The oral rinse sample was inoculated onto the Sabouraud dextrose agar with Chloramphenicol and was incubated at 37°C for 3-4 days. The Colony forming Unit of *Candida* was compared among diabetic and healthy controls. The *Candida albicans* were identified by Germ tube formation. The *Candida albicans* isolates were subjected to Biofilm assay, Antifungal susceptibility Test, Haemolysin assay, Haemolysis degree and phospholipase assay. Result: This study reported 31.5% prevalence of oral *Candida*. The *Candida* carriage in CFU of diabetic population was statistically significant ($p < 0.05$). The maximum isolates were found to be Biofilm producers. There was significant association between Gutkha consumers with oral *Candida* carriage. The study suggests that there is higher colonization of *Candida* in diabetic populations than in healthy population. The result also concludes that frequency of *Candida* in Oral cavity of Gutkha consumers was also higher ($p < 0.05$). All isolated strains of *Candida albicans* were tested for antifungal susceptibility testing and 76.19% were found to be Resistant to Fluconazole and 50% were found to be resistant to Amphotericin B. There was statistical significance in Biofilm formation and fluconazole Drug resistance. Conclusion The findings indicated highest colonization of oral *Candida* in diabetic population and in Gutkha consumers. The greatest numbers of isolated *Candida albicans* were biofilm producer which showed greater frequency of Fluconazole drug resistance.

Background

Candida albicans is known to be normal flora of human body harboring skin, mucosal cavity oral, vaginal mucosa and known to colonize 60% of healthy population [1]. *Candida albicans* is dimorphic fungi isolated most commonly from Biofilms of medical devices and human tissue [2]. Studies have suggested that *Candida albicans* are associated with number of opportunistic infections in immunocompromised patients like in HIV patients, cancer patients and diabetic patients etc. [3].

In diabetes mellitus type-II there has been known greater incidence of oral candidiasis which could be associated with immune dysfunction caused by high glucose concentration in blood, tissue and saliva [4]. Individuals with diabetes were found to have higher *Candida* carriage rate than the non-diabetics, presumably due to increased *Candida* growth with high glucose levels in saliva, blood and neutrophil dysfunction [5].

Candida has known to be opportunistic pathogen under immunosuppression and tobacco chewing conditions [6, 7]. It is suggested that individuals chewing *Gutkha* are susceptible to oral *Candida* infections than non-chewers [8]. The greater prevalence and colonization of *Candida* in *Gutkha* consumers could be due to the presence of nicotine and hydrocarbons such as polycyclic aromatic hydrocarbons as a nutritional source for Oral yeast facilitating its growth [7,8]. Chewing Tobacco that includes *Gutkha*, Betel quid (BQ) which is common habit in South Asian nations like in India, Pakistan, Bangladesh, Sri Lanka and Nepal [9]. *Candida albicans* pathogenesis is explained by its host defense mechanism, adherence, and production of tissue degrading hydrolytic enzymes like protease, phospholipase and haemolysin and role in biofilm production on host tissue and in medical devices [10]. Biofilm production is associated with the role of fungi to evade host immune function and overcome adhesions to host cell molecules to resist antifungal therapy making the treatment of infection more complicated [11].

Candida albicans exhibits hemolytic activity when grown on glucose-enriched blood agar [12]. In humans, most of the iron is located intracellular as ferritin or as heme-containing compounds. It is concluded that *C. albicans* expresses a hemolytic factor which allows it to acquire iron from host erythrocytes [13].

Materials And Methods

Research design: The cross sectional lab based study was carried out in microbiology laboratory of Central campus of Technology, Hattisar from June 2018 to November 2018. During the study 200 oral rinse samples were analyzed. All the work concerning this research was carried out in microbiology laboratory in Central Campus of Technology, TU, Hattisar, Dharan. The different oral rinse samples analyzed were from different potential risk population (50-barbers, 50-municipal waste workers, 50-diabetic and 50-healthy individuals). Ethical approval was obtained from Nepal Health Research Council, Kathmandu. The research participants were informed about the procedure of sample collection and the informed consent was obtained in written form. All the participants without oral lesions were included in the study. The Healthy population was selected by Nutritionist on basis of physical fitness, good oral hygiene whereas Diabetic population was selected on basis of blood glucose level obtained from their medical history. All the socio-demographic information like brushing habit, *Gutkha* chewing e.t.c was obtained from research participants through Schedule.

Inclusion criteria

Group I patients: In the inclusion criteria, 50- participant having DM without any other oral lesions with following criteria were included in the study. Participants who have not received antibiotic and corticosteroid therapy before 4 weeks were included in study.

1. Random blood sugar (RBS) ≥ 200 mg/dl or
2. Fasting blood sugar (FBS) > 126 mg/dl.

Group II (Control group): 50-People who did not have DM or any other systemic illness, who were nutritionally fit, without any clinical signs of diseases, without any clinical medication were included as Healthy control.

Group III: It included 50-Barber population across the city.

Group IV: It included 50-Municipal waste worker of Dharan Sub metropolitan city.

Exclusion criteria

The people who never met above criteria were included under exclusion criteria

Sample collection

Ten ml of sterile saline were allowed to be rinsed for 1 minute and inoculated in a broader capped sterile container. The oral rinse sample for study were collected from the sample population and transported to microbiology laboratory maintaining cold chain .All the collected samples were labeled with participant's identification number. In case of delay, the sample was usually stored at 4°C in the refrigerator.

Processing of oral rinse in SDA

An aliquot of 50 microliter of oral rinse sample were inoculated in SDA (HiMedia, Mumbai, India) with chloramphenicol (0.05 g/l) and incubated at 37°C for 24-48 hours. Pure culture was identified by colony characteristics, grams staining. The number of colony was counted by Colony Counter and expressed as CFU/ml [14, 15].

Assessment of *Candida* colonization by Quantitative Enumeration

Number of colonies formed after incubation were counted by colony counter counted and multiplied with a factor of 20 to get the colonies in 1 ml of a subject's sample [15].

Number of colonies contained in 50 µl of sample = n

Therefore the number of colonies in 1000 µl

(1 ml)

= n x 1000/50

= n x 20

Identification of *Candida albicans*

Germ tube and Chlamydospore formation was evaluated as described by Beheshti et al (1975) [16]. Germ tube test: The pure isolated colony of *Candida albicans* was dispensed in 0.5 ml of serum and incubated at 37°C for 2 hours. After incubation the aliquote was taken in a clean slide and was observed under microscope for the formation of Germ-tube.

Chlamydospore formation test: The pure isolated colony of *Candida* that could form Chlamydospore in Corn Agar was identified as *Candida albicans*.

Hemolysin Assay

Haemolysin activity was evaluated according to Luo et al 2001[13]. Hemolysin production by *Candida albicans* was performed by inoculation overnight culture of yeast on Sugar-enriched sheep blood agar as described by Manns et al (1994) [13]. The Blood base agar media was prepared by adding 5-7 ml of fresh blood to Saboured Glucose Agar with 3% glucose. 10 µL of the yeast inoculum was placed at the centre of the plates. The plates were incubated at 37°C in 5% CO₂ for 48 hours. Hemolytic Index (Hz value) which represents the Hemolysin production is the ratio calculated by dividing the total diameter of the colony plus the translucent halo by the diameter of the colony.

Screening *Candida albicans* for production of phospholipase

The phospholipase test was done according to Samranayakae et al (1984) [17]. Egg yolk agar media was inoculated by 2 µL of the inoculum and allowed to dry at room temperature. The plates will be incubated at 37°C for 3-4 days. *P_z* will be measured by dividing the diameter of the colony by the sum of diameter of the colony and the zone.

Screening impact of diabetes in hemolytic activity

The hemolytic activities were tested on Saboured dextrose Broth liquid media (SDB) containing 7% defibrinated human blood according to Malcok et al (2009) [12]. One was supplemented with 3% glucose and the other without glucose. *Candida albicans* culture was inoculated and incubated for 48 hrs. The hemolysis in the media was detected spectrophotometrically by measuring the amount of released hemoglobin and compared with a standard hemolysate which was prepared prior to testing. The degree of hemolysis (percentage value) by an individual strain was calculated according to the following formula below: (Absorbance of supernatant media at 540 nm/Absorbance of standard hemolysate at 540 nm X 100).

Biofilm Assays

Microtitre plate method

The quantification of biofilm was performed according to Christensen et al (1985) [18]. In this method, 5 ml of overnight culture of *Candida albicans* was prepared. Then 100 microliter of diluted culture was inoculated in a sterile 96-well polystyrene well plates well containing TSB with glucose. The plate was incubated at 37°C for 24 hours for biofilm production. The unbound cell was discarded and washes several times. 125 µl of 0.1 % crystal-violet solution was added and left for 10-15 mins incubation. The plate was washed and left inverted for dry. The quantitative Determination was performed by solubilizing the biofilm by adding 125 µl of 30% acetic acid to each well and incubated the plate for 10-15 mins at room temperature and was transferred to another microtitre plates and reading the absorbance at 570 nm by ELISA plate Reader. The interpretation of biofilm production was done according to the criteria of Stepanovic et al. (2007) [19].

Average OD	Value Biofilm production
$\leq \text{ODc} / \text{ODc} < \sim \leq 2\text{x ODc}$	Non/weak
$2\text{x ODc} < \sim \leq 4\text{x ODc}$	Moderate
$> 4\text{x ODc}$	Strong

Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control.

Tube method

A qualitative assessment of biofilm formation was done as described by Christensen et al (1985)[18]. The TSB glu (10 mL) was inoculated with a loop full of *Candida albicans* from overnight culture plates and incubated for 24 hours at 37°C. The tubes was decanted and washed with PBS (pH 7.2) and dried. Then the tubes were stained by 0.1% crystal violet. Stain was removed by deionized water. Tubes were then dried in inverted position for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not considered biofilm formation. An experiment was repeated for three times.

Congo red Agar Method (CRA)

The *Candida albicans* culture was streaked on surface of Congo Red Agar and incubated at 37°C for 24-48 hours (Freeman et al 1989) [20]. Black coloured colonies with dry crystalline consistency interpreted as positive biofilm producing strains. Red coloured colonies- interpreted as negative for biofilm production.

Antifungal Susceptibility Testing

All *Candida albicans* isolated from samples were subjected to in-vitro antifungal susceptibility test by Kirby-Bauer disc diffusion method as recommended by CLSI 2010 [21]. In this study the antifungal used were Amphotericin-B (100 Units) and fluconazole 10 (mcg) (HiMedia, Mumbai India). In this study, the most preferred class of antifungal agents like polyenes and azoles was included; amphotericin-B (100 Units) and fluconazole (25 µg/mL), (HiMedia, Mumbai, India) stored in at 4°C. Yeast inoculums were prepared by picking five distinct colonies of approximately one mm from each 24 hours old culture grew on Sabouraud Dextrose Agar (HiMedia, Mumbai, India) incubated at 35 ± 2°C. Colonies were suspended in 5 mL of sterile 0.85% Saline. Vortexed the resulting suspension and adjusted the turbidity to yield 1×10^6 - 5×10^6 cells/mL (i.e., 0.5 McFarland standard). A sterile cotton swab moistened with the inoculums suspension was used to apply to a 90 mm diameter plate containing Mueller-Hinton agar (HiMedia, Mumbai, India) supplemented with 2% glucose and 0.5 µg/mL methylene blue (GM-MH agar medium). The plates were allowed to dry for 5-15 minutes then after the disks were placed in the center of the agar plate. The plates were incubated for 18-24 hours at 37 ± 2°C and the slowly growing isolates will again read after 48 hours incubation. Zone sizes were measured in millimeters with Zone Scale (HiMedia, Mumbai, India); the zone diameter to the nearest point at which there was influential reduction in growth was taken into consideration. The organism was considered as resistant, intermediate or sensitive based on the standard interpretative chart according to CLSI standard (HiMedia Company, 2010).

Statistical Analysis

The data was analyzed using statistical Package for Social Sciences (SPSS) version 16.0. Mann Whitney U test was carried out to calculate CFU counts, Chi Square (χ^2) were used for statistical analyses. The p value of equal or less than 0.05 was used for statistical significance.

Results

Analysis of *Candida* Carriage between Diabetic cases and Healthy controls

Candida carriage expressed in CFU/ml was comparatively studied in both Case and control groups using Mann Whitney U test. The result of statistical analysis of *Candida* carriage is shown in Table 1 (Supplementary Files).

Prevalence of *Candida* in Population

The overall prevalence of *Candida* species was 31.5 % whereas the prevalence of *Candida albicans* only was 21 %. The result of prevalence of *Candida* yeast in population is shown in Figure 3.

Comparative study of *Candida albicans* isolated from different populations

Candida albicans were isolated from Diabetic population 29 followed by Barbers 15, Municipal Waste worker 6 and Healthy populations 13 respectively. The highest percentage of *Candida albicans* was

isolated from Diabetic population 16 (55.17%), followed by Barbers 15 (100%), Healthy population 6 (46.15%) and Municipal waste worker 5 (83.33%). The Comparative study of *Candida albicans* isolated from different Populations is shown in Table 2 (Supplementary Files).

Antifungal susceptibility pattern of *Candida albicans* isolates

It was found that 76.19 % (32/42) were Fluconazole resistant. Other antifungal agent was used to identify the susceptibility and resistant pattern of positive isolates and it was found that 50 % (21/42) were Amphotericin Resistant. Antifungal Susceptibility Test of isolated *Candida albicans* is shown in Table 3 (Supplementary Files).

Phospholipase and Haemolysin assay

The P_z value of isolated *Candida albicans* ranged from 2 to 2.95 for all the isolates and 2.01 to 2.6 for isolates from Diabetic group and 2.09 To 2.89 for control groups. The mean P_z value in Diabetic population was 2.11 and in Control groups was 2.52. The P_z Values of isolates are shown in Table 4 (Supplementary Files). The H_z values ranged from 5 to 18 for Diabetic groups and 3.45 to 16 for Control Groups. The mean H_z value in Diabetic group was 9.28 and in Healthy controls were 7.91. The Haemolysis degrees of isolates are shown in Table 5 (Supplementary Files).

Biofilm assay by microtitre plate method

The 96-well microtitre plate method for biofilm formation was carried out. The overall biofilm producers were 69.04 % and 35.96 % were non biofilm producer. The strong biofilm producer was 2 (4.76 %), moderate biofilm producer was 27 (64.28%) and Weak/none biofilm producer were 13 (30.96%). Biofilm assay by microtitre plate method is shown in Table 6 (Supplementary Files).

The Biofilm formation with Antifungal Drug Resistance is shown in Figure 5.

Sensitivity and Specificity of Biofilm Screening Methods

The microtitre plate method was found to be most efficient standard method for studying biofilm formation as compared to tube method and Congo red agar method. The parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were calculated and are shown in Table 7 (Supplementary Files).

Discussion

The present Study was carried out at Microbiology and Molecular Biology Laboratory of Central Campus of Technology, Dharan. During the study period a total of 200 oral rinse samples were collected from

three potentially risk populations (50 barbers, 50 municipal waste workers, 50 diabetic populations) and 50 healthy populations (Control). In the present study the overall prevalence of *Candida* species in sample population was 31.5% (63/200) and the prevalence of *Candida albicans* was 21% (42/200). In this study, *Candida albicans* were isolated from 29 (69.04%) male population and 13 (30.96%) female population. Majima et al (2014) reported 17.8% oral candida carriage in male and 18.8% oral *Candida* carriage in female [22]. Javed et al 2009 reported greater prevalence of *C. albicans* in oral cavity of denture wearing males (74%) than in female (23%) with diabetic mellitus Type II disease. Loster et al (2016) reported that the infection free individual were greater in male than in female and statistically significant relationship between intensity of yeast and gender [23].

In our study the maximum frequency of organism was found in between age group of 40-50 years. The association of *Candida* in different age groups was found to be statically insignificant ($p>0.05$). Lockhart et al (1999) found the increase in oral candida carriage was reported by with age possibly due to diminished natural defenses [24]. In our study the prevalence of yeast in elderly age group was found to be least possibly due to least sample population from old age group. There was no significant association between Age and prevalence of *Candida albicans*.

This study reported 31.5 % prevalence of oral *Candida*. Out of 63 positive Samples of *Candida*, 42 isolates were known to be *Candida albicans*. Around 85-90 % of diabetic patients are diagnosed by Type-2 Diabetes. In Diabetic patients the salivary dysfunction such as Xerostomia, tooth decay, decreased salivary flow and periodontal diseases are common (Ship 2003) [25]. Diabetic patients are more susceptible to oral Candidiasis due to increased salivary glucose, low secretion of saliva, impaired chemotaxis and defect of phagocytosis (Kadir et al 2002)[26]. Many Risk factors are associated with colonization by *Candida*. Poor oral hygiene, diabetic conditions, immunosuppressive therapy in Cancer disease, intake of *Gutkha* has shown increasing prevalence of *Candida* species (Guggenheimer et al 2000)[27]. Statistically significant increase in *Candida* carriage in terms of colony forming units were reported in patients with Diabetes mellitus than in control Groups, $p<0.05$ with CFU/ml ranging from 0 to 1700. This result is similar too many other studies that explain the higher susceptibility of *Candida* to colonize oral cavity of diabetic patients. The study conducted by Lamichhane et al (2015) showed significant increase in *Candida* CFU in Diabetic patients than in Controls [28]. Host factors which contribute to oral Candidal carriage in Diabetes are known to be Candidacidal activity of neutrophils in presence of Glucose. In diabetic patients with Candidiasis the Polymorphonuclear leucocytes (PMN) produces less free oxygen radicals exhibiting reduced phagocytosis, bactericidal activity and chemotaxis. This may confer Diabetic patients more susceptible to Oral *Candida* infections (Soysa et al 2005) [29]. Mohammadi F et al 2016 found 55% Diabetic patient to carry *Candida* sp. in oral cavity which *C. albicans* was abundant species. Belazi et al (2005) isolated candida sp. from oral cavity of 64% diabetic and 40%

control population. Many researchers conducted throughout the world have reported cases of oral candidiasis in diabetic patients commonly known as oral thrush [3].

Pathogenic microorganisms are capable of acquiring iron for survival and establish infection in host that addresses its pathogenicity. Since there is little iron human body, the most microorganisms derive iron from hemoglobin. So to destroy hemoglobin, they secrete enzyme like haemolysin (Malcok et al 2009) [12]. The mean hemolytic degree of *Candida albicans* in human blood with Glucose (SDBwG) was 58.68% and in human blood without glucose (SDBwoG) was 48.74%. We reported that *Candida albicans* exhibited higher hemolytic degree. Malcok et al (2009) reported in his study that most species of *Candida* exhibited hemolytic activity in 3% glucose enriched medium and hence suggested the parallel combination of Diabetic condition with pathogenesis of *Candida albicans* [12]. In-vitro analysis of hemolytic degree of *Candida albicans* in presence and absence of glucose level strongly explains the increased blood sugar level increases the Hemolysin activity of *Candida albicans*. This study suggests the pathogenic role of *Candida albicans* in Diabetic mellitus and supports the concept of susceptibility of *Candida* colonization and Candidiasis in Diabetic patients. The Haemolysin Index of *Candida albicans* isolated from Diabetic patients was found higher than in Control population.

The Pz value of isolated *Candida albicans* ranged from 2.01 to 2.6 for isolates from Diabetic group and 2.09 To 2.89 for control groups. The mean Pz value in Diabetic population was 2.11 and in Control groups were 2.52. There was no significant difference in phospholipase activity in Diabetic and control groups ($p > 0.05$). Tsang et al (2007) reported no significant difference among phospholipase activity among *Candida albicans* isolated from Diabetic and Control groups. Tsang et al (2007) reported higher proteinase and Haemolytic activity from Diabetic patients with no significant difference in phospholipase activity from test and control groups [29]. Sachin et al (2012) reported 94.8% *C. albicans* exhibiting higher haemolysin activity and 92.3% exhibiting phospholipase activity [30]. In our study all strains of *C. albicans* exhibited haemolysin activity and only 68% *C. albicans* exhibited phospholipase activity. In our study there was significant difference in Hemolysin activity among Diabetic and control groups ($p = 0.049$).

Candida has known to be opportunistic pathogen under immunosuppression and tobacco chewing conditions [6, 7]. It is suggested that individuals chewing *Gutkha* are susceptible to oral *Candida* infections than non-chewers [8]. Chewing Tobacco that includes *Gutkha*, Betel quid (BQ) which is common habit in South Asian nations like in India, Pakistan, Bangladesh, Sri Lanka and Nepal [31]. Betel quid is mixture of areca-nut, lime enveloped in piper betel leaf whereas *Gutkha* is found in Sachet [32]. The possible Explanation for greater *Candida* colonization in *Gutkha* 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 [7.8]. In our study, higher prevalence of *Candida* carriage was found in *Gutkha* chewers. The *Candida* colonization and *Gutkha* consumption was statistically significant

, $p < 0.05$. Similar study by Abduljabbar et al (2017) reported a significant difference in Oral *Candida* carriage in *Gutkha* chewer and Betel Quid chewer suggesting the susceptibility to oral candidiasis in those risk groups than in control groups[8]. However Javed et al (2014) showed no significant difference in oral *Candida* colonization among *Gutkha* and Betel quid chewer [6]. This contradiction in our study could be due to fact that Oral rinse sample were obtained for complete enumeration of *Candida* from oral cavity. The prevalence of oral *Candida* carriage in healthy population having good oral hygiene was low as compared to others. The healthy populations included individuals without physical complications and diseases, proper BMI, and no smoking, chewing and Drinking Habit. The lower frequency of *Candida* carriage in healthy might be due to maintenance good oral hygiene and practice. We can at least explain that being a part of normal flora the colonization of *Candida* is associated even with poor oral hygiene.

All isolated strains of *Candida albicans* were tested for antifungal susceptibility testing by using Kirby Bauer disk diffusion method 76.19% were found to be Resistant to Fluconazole and 50% were found to be resistant to Amphotericin B. It was observed that emergence of drug resistance in *Candida albicans* is a major global issue which persuade for new search of therapeutic agents for eradicating the infections. The emerging Drug Resistance is has become global burden for treating the infections. *Candida* infection has been increasing in immunocompromised, over aged, patients receiving cancer chemotherapy and organ transplantation.

Biofilm is the surface-attached microbial community that contributes virulence factor. Biofilm helps *Candida* to escape host defense and develop resistance to antimicrobial agents. Over last two decades the burden of *Candida* infection throughout the world has been rising with drug resistance. Diabetes mellitus patients, immuno-compromised patients like HIV patients, antibiotic users, IV users, *Gutkha* consumers etc. are at risk to *Candida* infection. Biofilm of *Candida* is made up of layers of cells embedded in matrix of extracellular polymeric materials (Khatri et al 2015) [33].

In our study the strong, moderate and weak biofilm producers by microtitre plate method were 4.7%, 64.2% and 30.9% respectively. The strong, moderate and weak biofilm producers by TM method were 4.7%, 38% and 57.1% respectively. The strong, moderate and weak biofilm producers by CRA method were 2.3%, 35.7% and 61.9% respectively. Number of false positive and false negative was reported in the comparison. It was difficult to discriminate strong, moderate and weak biofilm producers in Tube Method and Congo Red Agar method due to phenotypic variations. Sensitivity and specificity of TM was found to be 71.4% and 62.8% respectively with accuracy of 64.2%. For Congo red agar method the sensitivity and specificity was found to be 27.7% and 16.6% with accuracy of 21.4%. The statistical analysis of screening were similar even in our findings which supports different other similar findings done before. Hassan et al (2011) concluded Microtitre method as gold standard technique for screening biofilm as

compared to Tube method and Congo Red Agar [34]. Even in our study The Microtitre method was considered efficient method for quantitative screening of biofilm in comparison to TM and CRA methods.

In our study 4.7% of isolated *Candida albicans* were strong biofilm producers, 64.2% were moderate and 30.9% were weak or non-biofilm producer. In overall the biofilm forming *Candida albicans* were 69% and non-biofilm producers were 30.9%. In our study the maximum frequency of *Candida albicans* were biofilm producers and even maximum biofilm producing *Candida albicans* were resistant to antifungal drugs. This association may explain the ability of biofilm production with drug resistance in the organism exhibiting its strong virulence factor and making failure of antifungal drugs. Mohammadi et al (2016) reported that biofilm formation and overgrowth of *Candida* is found more in diabetic patients [35]. Similar to it even in our study the antifungal resistance pattern of *Candida albicans* showed highest resistance to fluconazole whereas Amphotericin B also showed good sensitivity.

The biofilm formation and Fluconazole resistance was statistically significant $p < 0.05$, whereas the biofilm formation and Amphotericin B drug resistance was not significant. Amphotericin B showed a greater Sensitivity towards *Candida albicans*. Weak biofilm producing *Candida albicans* isolates showing resistance towards Amphotericin B could be due to efflux pump other than biofilm. Most studies suggest that Amphotericin B displays a good activity against *Candida* biofilms. According to study conducted by Mahmoudabadi, Zarrin et al (2014) the 100% isolates of *Candida albicans* were biofilm producers where 65% showed Sensitivity towards Amphotericin B and only 26.7% Sensitivity towards Fluconazole [36]. Similar findings were observed even in our study.

In Nepal, improper use of antibiotics drugs without doctor's prescription, haphazard selling and buying of antibiotic from local pharmacy and unnecessary use of drugs have been responsible for drug resistance to microorganisms. This uncontrolled use of drug has led to antifungal drug resistance in strains of microorganisms. Indiscriminate use of drugs in our community is a major cause for emergence of drug resistance. The proper availability of microbiological investigation and information would be necessary in search of appropriate antimicrobial agents to fight with drug resistance. Since for many years Fluconazole has been used for treatment of oral candidiasis but the emergence of drug resistance renders the search of alternative antimicrobial therapy.

The good oral hygiene and the reduced prevalence of *Candida* carriage was statistically significant, $p < 0.05$. In our study the total *Candida* carriage among healthy population was only 26 %. Majima et al (2014) found 18.3% oral *Candida* carriage among dental students and suggested that good oral hygiene responsible for elimination of *Candida* [22]. Therefore with overall analysis in potential risk groups and in

Healthy population, the oral *Candida* colonization shows a significant association with oral hygiene and serum glucose.

Higher prevalence of *Candida* in diabetic population and in poor oral condition alarms the oral health warnings. The absence of *Candida* in oral cavity may be due to maintenances of good oral health. Thus, oral carriage of *Candida* serves for health issues as oral candidiasis requires initial colonization by *Candida*.

Declarations

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Tables

Due to technical limitations, tables 1 - 7 are only available as a download in the supplemental files section.

Figures

Figure 1

Distribution of colony forming units (CFUs) among Diabetic cases and Healthy control.

Figure 2

Candida Carriage in CFU from Healthy (Left) and Diabetic case (Right)

Figure 3

Prevalence of Candida in Population

Figure 4

Hemolysis Degree exhibited by isolated Candida albicans in SDB with and without Glucose

A

B

C

D

Figure 4: Biofilm formation by different Techniques and Haemolysis Degree
 Biofilm formation by *Candida albicans* on Congo Red agar characterized by black colony (A). Biofilm formation by *Candida albicans* on Tube (B). Biofilm formation by *Candida albicans* on Microtitre plate (C). Hemolysis Degree exhibited by *Candida albicans* on SDB with Glucose (Right) and SDB without Glucose (Left) (D).

Figure 5

Biofilm formation by different Techniques and Haemolysis Degree Biofilm formation by *Candida albicans* on Congo Red agar characterized by black colony (A). Biofilm formation by *Candida albicans* on Tube (B). Biofilm formation by *Candida albicans* on Microtitre plate (C). Hemolysis Degree exhibited by *Candida albicans* on SDB with Glucose (Right) and SDB without Glucose (Left) (D).

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