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Original Article

Antifungal susceptibility test of biofilm-producing pathogenic Candida albicans isolated from oral cavity of type II diabetic patients and non-diabetic individuals

Bijay Kumar Shrestha[1], Jenish Shakya[1], Manita Tumbahangphe[1], Hemanta Khanal[1], Bidhya Dhungana[1], Romika Shrestha[1], Kabita Dhakal[1], Sujata Chauhan[1], Santoshi Ghimire[1], Jyoti Limbu[1], Kaushila Limbu[2]

[1]*Department of Microbiology, Central Campus of Technology, Tribhuvan University, Hattisar, Dharan, Nepal.*
[2]*Department of Microbiology, Kantipur College of Medical Science, Tribhuvan University, Kathmandu, Nepal.*

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ABSTRACT

Background: Candida albicans are found in the mucous membranes of the respiratory, gastrointestinal, and female genital systems as part of the natural flora. Diabetic people are more susceptible to C. albicans infections due to elevated blood glucose and the immune system's failure in fungus eradication. This study aimed to look at Candida carriage and antifungal susceptibility testing of biofilm-producing C. albicans isolated from the oral cavity of type II diabetic patients and non-diabetic individuals.

Materials and methods: This was a cross-sectional analytical laboratory-based study carried out in Dharan Sub-Metropolitan city from June 2018 to November 2018. The 10 mL oral rinse was collected from 50 diabetic patients and 50 healthy control participants. Isolation, identification, biofilm assay, and antifungal susceptibility test of C. albicans were performed by the conventional microbiological procedure. Statistical analysis was used to determine the association between variables.

Results: The Candida carriage was significantly higher in diabetic patients 58% (29/50) than in healthy (control) groups 26% (13/50) (p=0.001). In the antifungal susceptibility test of C. albicans isolated from diabetic patients, 18.75% isolates were sensitive, 81.25% isolates were resistant to fluconazole, 43.75% isolates were sensitive, and 56.25% isolates were resistant to amphotericin-B. The biofilm formation and fluconazole drug resistance were found to be statistically significant (p=0.029).

Conclusions: The findings concluded the highest colonization of oral Candida in diabetic patients than in healthy (control) individuals. Emerging antifungal drug resistance is even associated with biofilm formation, which requires the importance of displaying an antifungal susceptibility profile before antifungal therapy.

Correspondence:

Bijay Kumar Shrestha,

Department of Microbiology, Central Campus of Technology, Tribhuvan University,

Hattisar, Dharan, Nepal.

ORCID ID: 0000-0002-6542-829X

E-mail: interfacebj@gmail.com

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INTRODUCTION

C. albicans is known to be normal flora of the human body harboring skin, mucosal cavity, oral cavity, vaginal mucosa and known to colonize 60% of a healthy population.[1] C. albicans is dimorphic fungi isolated most from biofilms of medical devices and human tissue.[2] Studies have suggested that C. albicans are associated with a number of opportunistic infections in immunocompromised patients like HIV patients, cancer patients, diabetic patients, etc.[3] In diabetes mellitus type–II there has been a known greater incidence of oral candidiasis which could be associated with

Table1: Candida carriage in diabetic patients

Parameter	Study group (n)	Candida carriage	Prevalence of C. albicans	Minimum CFU/mL	Maximum CFU/mL	Mean CFU/mL	p-value
CFU/ml	Diabetic patients (50)	58% (29/50)	32% (16/50)	620	1700	599.80	p=0.001
	Healthy Control (50)	26% (13/50)	12% (6/50)	220	840	127.4	

immune dysfunction caused by high glucose concentration in blood, tissue, and saliva.[4] In addition, individuals with diabetes were found to have a higher Candida carriage rate than the non-diabetics, presumably due to increased Candida growth with high glucose levels in saliva, blood, and due to neutrophil dysfunction.[5]

Lactoferrin, sialoperoxidase, lysozyme, histidine-rich polypeptides, and specialized anti-candida antibodies are antimicrobial proteins found in saliva that interact with the oral mucosa and prevent Candida overgrowth.[6] Drugs that reduce cellular immunity and phagocytosis, such as inhaled steroids, have been demonstrated to increase the incidence of oral candidiasis.[7] Primary and secondary candidiasis are two types of oral candidiasis. Acute (pseudomembranous and erythematous), chronic (pseudomembranous, erythematous, and hyperplastic), and Candida-related lesions are the subtypes of primary oral candidiasis, whereas secondary oral candidiasis is defined as involvement of other body organs in addition to the mouth.[8] Factors like smoking, diabetes, Cushing's syndrome, immunosuppressive conditions such as HIV infection, malignancies such as leukemia, and nutritional deficiencies like vitamin-B deficiencies have been associated with oral candidiasis.[9] Candida are opportunistic pathogens that only infect the mouth when the host has an underlying susceptible condition.[10]

C. albicans pathogenesis is explained by its host defense mechanism, adherence, and production of tissue degrading hydrolytic enzymes like protease, phospholipase, hemolysin, and role in biofilm production on host tissue and in medical devices.[11] 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.[12]

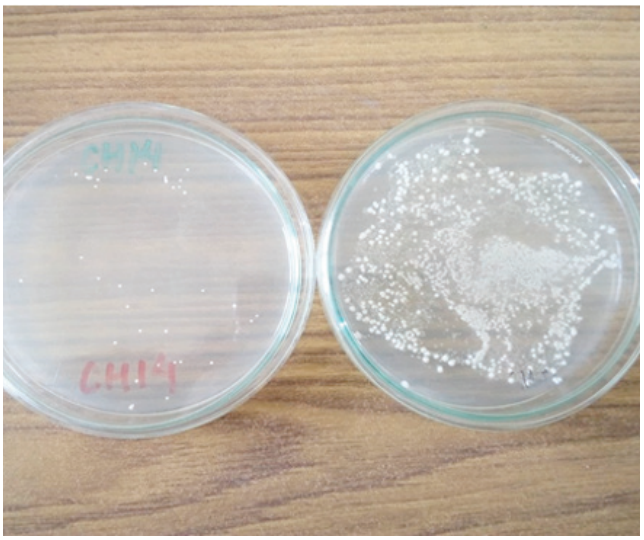

Figure 1: Colonies of Candida from Healthy controls (left) and Diabetic patients (right).

In Nepal, there have been few investigations on the prevalence of oral candidiasis in diabetic patients. The availability of antimicrobials over the counter, combined with a high rate of empiric treatment, contributes to Nepal's rising antimicrobial resistance. The study from Nepal reports Diabetic patients are more prone to fungal infection than

Table 2: Antifungal susceptibility test of isolated C. albicans

Antifungal agents	Diabetic patients		P - Value	Healthy Control		P - Value
	Sensitive	Resistant		Sensitive	Resistant	
Fluconazole (25µg)	3 (18.75%)	13 (81.25%)	0.001	4 (66.66%)	2 (33.33%)	0.248
Amphotericin-B (100U)	7 (43.75%)	9 (56.25%)	0.480	5 (83.33%)	1 (16.66%)	0.021

Table 3: Biofilm assay of isolated oral C. albicans

Parameter	Biofilm formation	Prevalence in diabetic (%)	Prevalence in control (%)	p-value
CFU/ml	Strong	7 (43.75%)	-	0.001
	Moderate	3 (18.75%)	2 (33.34%)	
	Weak	2 (12.5%)	-	
	None	4 (25%)	4 (66.66%)	
	Total	**16**	**6**	

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Table 4: Biofilm formation and antifungal drug resistance pattern of oral C. albicans

Group	Antifungal agents	Biofilm producing C. albicans resistant to	Biofilm producing C. albicans sensitive to	Biofilm non-producing C. albicans resistant to	Biofilm non-producing C. albicans sensitive to	Total	p-value
Diabetic patients	Fluconazole	10 (62.5%)	2 (12.5%)	1 (6.25%)	3 (18.75%)	16	0.029
	Amphotericin-B	5 (31.25%)	7 (43.75%)	2 (12.5%)	2 (12.5%)	16	0.771
Healthy Control	Fluconazole	2(33.33%)	-	3 (50%)	1(16.66%)	6	0.439
	Amphotericin-B	1(16.66%)	1(16.66%)	2 (33.33%)	2(33.33%)	6	1.00

Figure 2: Antifungal susceptibility test of C. albicans.

non-diabetic participants. In addition, drug resistance has been on the rise in recent decades because of the widespread usage of antifungal medicines.[13] In Nepal, the true burden of fungal infections has yet to be determined or is just in the early stages of being determined. There has been little research on the frequency of fungal opportunistic infections in diabetic patients.[13,14] However, none of the investigations looked at fungal opportunistic infections and antibiotic susceptibility with the biofilm-forming ability of pathogens at Dharan City. Therefore, this study was conducted to study the Candida carriage and antifungal susceptibility of biofilm-producing C. albicans isolated from the oral cavity of Type II Diabetic patients and non-diabetic individuals (healthy controls) of Dharan, Nepal.

MATERIALS AND METHODS

Comparative study of C. albicans sensitivity to fluconazole and amphotericin B in the oral cavity of diabetic and non-diabetic patients. From June to November 2018, a cross-sectional analytical (comparative) laboratory-based study was conducted in Dharan Sub-Metropolitan city. Dharan is a city in the Sunsari district of Province No. 1, with latitude 26° 49′ 12″ N and longitude 87° 18′ 0″ E with an elevation of 349 m. In total, 50 diabetic patients and 50 healthy controls were enrolled in the study after computing sample size n=100 by formula n= N/1+N(e)2, where N= Population size of 135, e= 5% level of precision. Thus, 100 oral rinse samples from the participants were examined.

Inclusion criteria: The inclusion criteria for the study included group-I (50-diabetic patients) having diabetes mellitus type II with random blood sugar (RBS) ≥200 mg/dL and Fasting blood sugar (FBS) ≥126 mg/dL, without any other oral lesions, who have not received antibiotic and corticosteroid therapy before 4 weeks were included in the study. The diabetic patients who visited the tertiary health care center of Dharan were selected by simple random sampling following lottery methods. Five tertiary health care centers were selected for this study and from each, 10 diabetic patients were being enrolled.

Exclusion Criteria: Participants in Group-II (50-Control group) did not have diabetes mellitus or any other systemic illness, did not have any clinical symptoms of disease, and did not take any clinical medicine. The healthy controls were selected from a group who were declared nutritionally fit by a dietitian, and physically healthy by a general physician after medical examination. They were also chosen at random using a basic random sample method based on the lottery method. Exclusion criteria were applied to those who have never matched the aforesaid criteria.

Culture and identifications of C. albicans: About 10 mL of sterile saline were allowed to be rinsed for 1 minute and inoculated in a broader capped sterile container. The oral rinse sample was collected and transported to the microbiology laboratory of Central Campus of Technology, Hattisar maintained in an ice-cold box. All the collected samples were labeled with the participant's identification number. On arrival at the laboratory, the samples were vortexed properly and processed within 2 hours. An aliquot of 50 µl of oral rinse sample was inoculated in Sabouraud dextrose agar (SDA) (HiMedia, India) with chloramphenicol (0.05 gm/l) and incubated at 37 °C for 4 days. The pure culture was identified by colony characteristics and simple staining. The number of the colony was counted by colony counter and expressed as CFU/mL.[15] Germ tube and chlamydospore formation was evaluated as described by Beheshti et al[16]. In 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, an aliquot was taken in a clean slide and was observed under a microscope for the formation of germ-tube. In chlamydospore formation test, the pure isolated colony of candida that could form chlamydospore in corn agar was identified as C. albicans.

Biofilm Assay of C. albicans: Biofilm quantification was carried out according to Christensen et al[17]. In this procedure,

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5 mL of C. albicans overnight culture in Sabouraud dextrose broth was formulated (HiMedia, India). After that, 25 µl of diluted culture was inoculated in a sterile 96-well polystyrene tissue culture plate (HiMedia, India) well with 100 µl tryptic soy broth (HiMedia, India) supplemented with 1% glucose. For biofilm formation, the plate was incubated at 37 °C for 24 hours. The unattached cell was removed after numerous washes in sterile phosphate buffer saline (pH 7.2) (HiMedia, India). A total of 125 µl of 0.1 percent crystal violet solution was added, and the mixture was incubated for 10-15 minutes. To fix the biofilms, the plate was washed and inverted for drying at 30 minutes for 35 minutes. The quantitative determination was carried out by solubilizing the biofilm in each well with 125 µl of 30% acetic acid (HiMedia, India), incubating the plate for 15 minutes at room temperature, and then transferring it to another tissue culture plate for reading the absorbance at 570 nm using an ELISA plate reader (Loncare LR-620 microplate reader, Medical Technology Co., Ltd.). The optical density (OD) of test wells was used to interpret the results. The experiment was repeated three times. The arithmetic mean of the absorbance of three wells was used to calculate the optical density (ODs) of each strain, which was then compared to the mean absorbance of negative controls (ODnc). Biofilm formation was classified as follows: no biofilm production (ODs≤ODnc), weak biofilm production (ODnc<ODs≤2. ODnc), moderate biofilm production (2.ODnc<ODs≤4. ODnc), and strong biofilm production (4.ODnc<ODs) as described by Stepanovic et al[18].

Antifungal susceptibility test of C. albicans: According to CLSI19, all C. albicans isolates were tested in vitro for antifungal susceptibility using the Kirby-Bauer disc diffusion method. The most used antifungal drugs, polyenes, and azoles, used in this investigation were amphotericin-B (100 units) and fluconazole (25 µg) (HiMedia, India). Picking five separate colonies of roughly one mm from each 24-hour old culture grown on Sabouraud dextrose agar (HiMedia, India) incubated at 37 °C yielded yeast inoculums. Five colonies were suspended in sterile 5 mL saline (0.85 %). The suspension was vortexed, and the turbidity was corrected to 0.5 McFarland standards. The inoculum suspension was applied to a 90 mm diameter plate containing Mueller-Hinton agar (HiMedia, India) supplemented with 2% glucose and 0.5 µg/mL methylene blue using a sterile cotton swab saturated with the inoculum suspension. The antifungal disks were placed in the center of the agar plate after the plates had dried for 5-15 minutes. The plates were incubated at 37 °C for 24 hours, and the slowly growing isolates were read again after 48 hours. The zone diameter to the nearest point at which there was an influential reduction in growth was measured in millimeters with the zone scale. Based on the usual interpretation chart, the organism was classified as resistant, intermediate, or susceptible.

Data collection: A questionnaire was used to collect information about the diabetes status, the date of sample collection, the sample site, the kind of sample, and the length of antibiotic therapy. The laboratory records were used to gather microbiological data. The research participants were informed about the sample collection technique and written informed consent was obtained.

Data management and statistical analysis: The data was imported into MS EXCEL 2010 and processed with SPSS 16.0, the statistical software for social sciences. The data were presented using frequency, percentages, and tables. Mann Whitney U test was used to see if there was a link between CFU counts in the test and control groups. To examine the relationship between dependent and independent variables, the Chi-square ($\chi 2$) test was utilized. The p-value less than 0.05 was statistically significant.

Ethical committee approval: Ethical approval (Reg. no. 296/2018) to conduct this study was obtained from Nepal Health Research Council, Kathmandu, Nepal.

RESULTS

In group-I, diabetic patients, 58% (29/50) carried Candida in their oral cavity with C. albicans prevalence of 32% (16/50). In group-II, healthy control, 26% (13/50) carried Candida in their oral cavity with C. albicans prevalence of 12% (6/50). The mean Candida CFU was significantly higher in diabetic patients (599.80 CFU/mL) than in the control group (127.4 CFU/mL). The Candida colony count among the diabetic and control group was found to be statistically significant (p=0.001) (fig. 1 and Table 1).

In the antifungal susceptibility test of C. albicans isolated from the oral cavity of diabetic patients, 18.75% isolates were sensitive, and 81.25% isolates were resistant to fluconazole. Similarly, 43.75% of isolates were sensitive, and 56.25% isolates were resistant to amphotericin-B. Similarly, in the antifungal susceptibility test of C. albicans isolated from the oral cavity of healthy controls, 66.66% isolates were sensitive, and 33.34% isolates were resistant to fluconazole. Similarly, 83.33% of isolates were sensitive, and 16.67% isolates were resistant to amphotericin-B (fig. 2 and Table 2).

Biofilm assay by tissue culture plate method reported 43.75% isolates strong biofilm producer, 18.75% moderate biofilm producer, 12.5% weak biofilm producer and rest 25% non-biofilm producer from diabetic patients. Biofilm assay by tissue culture plate method reported 33.34% moderate biofilm producer and 66.66% non-biofilm producer from healthy controls (p=0.001) (Table 3).

In diabetic patients, Biofilm producing C. albicans resistant and sensitive to fluconazole drug was found to be 62.5% and 12.5% respectively. The biofilm formation and fluconazole drug-resistant among isolates were statistically significant (p=0.029). Biofilm producing C. albicans resistant and

sensitive to amphotericin-B was reported to be 31.25% and 43.75% respectively. The biofilm formation and amphotericin-B drug-resistant among isolates was not found to be statistically significant (p=0.771). In healthy controls, Biofilm producing C. albicans resistant to fluconazole drug was found to be 33.33%. The biofilm formation and fluconazole drug-resistant among isolates were not statistically significant (p=0.439). Biofilm producing C. albicans resistant and sensitive to amphotericin-B was reported to be 16.66% and 16.66% respectively. The biofilm formation and amphotericin-B drug-resistant among isolates was not found to be statistically significant (p=1) (Table 4).

DISCUSSION

Diabetic patients are more susceptible to oral candidiasis when Candida species are present in their oral cavity.[20] Although Lactoferrin, sialoperoxidase, lysozyme, histidine-rich polypeptides and specific anti-candida antibodies all interact with the oral mucosa to prevent Candida overgrowth.[6] However, Biofilm formation and Candida species overgrowth are considerably higher in diabetic individuals.[21] Individuals with uncontrolled diabetes are known to be more prone to superficial, systemic infections, and oral candidiasis.[22] Salivary dysfunction such as xerostomia and periodontal disorders are frequent in diabetic individuals.[23] Due to increased salivary glucose, decreased saliva output, poor phagocytosis, diabetic people are more susceptible to oral candidiasis.[24]

In this study, a significant increase in Candida carriage in terms of colony-forming units was reported in patients with diabetes mellitus than in control groups, p=0.001 with CFU/mL ranging from 0 to 1700. This result is similar to many other studies that explain the higher susceptibility of Candida to colonize the oral cavity of diabetic patients. One study by Lamichhane et al[25] reported that diabetic individuals have significantly higher Candida carriage than controls. Host factors that contribute to oral Candida carriage in diabetes are known to be hyposalivation, deficient neutrophil activity. Saliva contains secretory immunoglobulin A and free secretory components that prevent oral Candida adhesion and colonization. Thus, hyposalivation breach the normal homeostatic mechanism that provides a niche for Candida colonization. In diabetic patients with candidiasis, the polymorphonuclear leucocytes produce less free oxygen radicals exhibiting reduced phagocytosis, bactericidal activity, and chemotaxis, which confer diabetic patients more susceptible to oral Candida infections.[25,26] The result of the present study agrees with previous studies by Mohammadi et al[21] and Shenoy et al[22] who reported a greater prevalence of Candida carriage in the oral cavity of diabetic patients than in control groups. Salivary glucose levels are linked to blood glucose levels, and diabetes patients have more oral Candida colonization than healthy people.[27] Increased salivary glucose was linked to an increased prevalence of oral Candida in diabetic individuals, according to a study by Balan et al[28]. Oral candidiasis in diabetes individuals has been described in numerous research conducted around the world.[22] The increased Candida proliferation in diabetes patients could be attributed to higher salivary glucose levels. Furthermore, the changed oral microbial habitats may have aided yeast colonization of the oral cavity in diabetic patients. The presence of Candida over an extended period could be a risk factor for oral candidiasis in diabetes individuals.[25,26]

In this study, antifungal drug resistance to both fluconazole and amphotericin-B was higher in C. albicans isolated from diabetic patients than from healthy control (Table 2). A study by Bhuyan et al[29] reported C. albicans isolated from the oral cavity of diabetic patients with the highest antifungal drug resistance towards fluconazole and amphotericin-B which was similar to the present findings. In agreement with this study, a study by Hedayati et al[30] reported C. albicans isolated from diabetic patients sensitive to amphotericin-B but resistant to fluconazole. Fluconazole resistance is a big problem because it is the most routinely used azole for both superficial and profound candidiasis.[31] Resistance to Amphotericin-B among C. albicans is also emerging in recent years and is known to be induced by deposition of the sterol intermediates in the drug-resistant strain interfering the activity of amphotericin-B.[32] The increasing incidence of fluconazole resistance among pathogenic C. albicans in Nepal is a sign of serious health concern. In the context of Dharan city, the fluconazole drug is widely marketed and used antifungal medication by the patients. The practice of irrational and indiscriminate use of antifungal drugs by patients has led to the serious problem of antifungal resistance. The emerging drug resistance has become a global burden for treating infections.

Microbial biofilm formation is associated with avoiding host immune action and overcoming adhesions to host cell molecules to withstand antifungal treatments, making infection treatment more difficult.[12] Biofilm of Candida is made up of layers of cells embedded in a matrix of extracellular polymeric.[33] In this study, biofilm-producing C. albicans was significantly higher among diabetic patients than in healthy control (p=0.001). Increased blood glucose levels in diabetes individuals may facilitate biofilm development.

Over the last two decades, the global burden of Candida infection has increased with drug resistance. Biofilm development and fluconazole resistance were statistically significant in this investigation (p=0.029); however, the biofilm formation and amphotericin-B drug resistance was not significant (p=0.771). A study by Lamichhane et al[14] reported that Biofilm producing Candida was more resistant towards commonly used antifungal drugs which agreed to the present study. Amphotericin-B showed greater sensitivity than fluconazole towards biofilm-producing C. albicans. Even Mahmoudabadi et al[34] reported that amphotericin-B has good effectiveness against candida biofilms. Non-biofilm producing C. albicans isolates showing resistance towards amphotericin-B could be due

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to efflux pump, genetic mutation, etc. The colonization of the oral cavity by pathogenic strains of microorganisms requires a sequence of different dependent and independent factors. In the case of diabetic patients, the hyposalivation increased salivary and blood glucose concentration are a few of many other multiple factors facilitating Candida carriage.[24] However, this study explains the relation of diabetes with Candida colonization. Prolific oral carriage of Candida serves important health issue as oral candidiasis requires prior colonization by Candida.

Enough socio-demographic information was not collected and Susceptibility towards other antifungal drugs was not tested.

CONCLUSION

In conclusion, the present findings report that diabetic patients are more susceptible to oral Candida carriage which is likely to contribute to oral candidiasis. The increasing burden of antifungal drug resistance among C. albicans might pose severe clinical challenges. Therefore, control over the indiscriminate use of antifungal drugs and selection of prophylactic antifungal agents after susceptibility testing can help to manage the infection.

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