Candida Species Diversity in Oral Cavity of Type 2 Diabetic Patients and their *In vitro* Antifungal Susceptibility

Abstract

Objective: To identify and compare the species variation and Colony Forming Units of the species and antifungal susceptibility from oral rinse samples of individuals in poorly-controlled, moderately-controlled and well controlled diabetes patients with control group. **Subjects and Methods:** Study group comprised of well-controlled, moderately-controlled and poorly controlled Type II diabetic patients grouped according to the level of glycated hemoglobin concentration with 50 patients in each group and 50 healthy individuals. Oral rinse samples were collected in a sterile container with phosphate-buffered saline and then transported immediately for various mycological investigations and antifungal susceptibility tests. Statistical analysis was performed. **Results:** There was a significant difference in frequency of *Candida* in poorly controlled diabetes when compared to moderately controlled diabetes, well controlled diabetes and normal patients ($P = 0.045$). A higher number of colony count was seen among poorly controlled diabetes than well controlled, moderately controlled and non diabetic subjects. A comparatively low number of non-albicans were seen in healthy individuals. *C. albicans* showed an increased resistance to fluconazole in DM patients in comparison to control group ($P = 0.001$). Other species showed a variable sensitivity pattern. **Conclusion:** The decreased immunity and change in oral habitat in diabetic patients creates a diversification in various species of *Candida*. These non albicans vary in their susceptibility and pathogenesis. A definite identification of these diverse species in the oral cavity of such patients and their susceptibility mandates proper management to avoid recurrence and drug resistance.

Keywords: *Antifungal susceptibility, Candida species, diabetes, resistance*

Lipsa Bhuyan,
Sahina Hassan[1],
Kailash Chandra Dash,
Abikshyeet Panda,
Shyam Sundar Behura, Sujatha Ramachandra

Department of Oral Pathology and Microbiology, Kalinga Institute of Dental Sciences, Kalinga Institute of Industrial Technology Deemed to be University, Bhubaneswar, Orissa, [1]Department of Microbiology, Late Baliram Kashyap Government Medical College, Jagdalpur, Chhattisgarh, India

Introduction

The prevalence of diabetes mellitus (DM) has increased dramatically worldwide with largest number of cases in India. It is a group of chronic diseases distinguished by cellular resistance to insulin action, insulin deficiency, or both, which results in hyperglycemia and other related metabolic disturbances.[1] The impaired innate immunity and acquired immunity in DM lead to the increase in infectious organisms.[2] Among various organisms causing opportunistic infections in oral cavity, members of the genus *Candida* are considered as most common commensals. Predisposing factors such as nutrition, decreased salivary function, change in pH of saliva, and high level of salivary glucose aid the overgrowth of *Candida* in the oral microenvironment.[3]

Candida albicans is the most common pathogen; however, there has been an upsurge in levels of nonalbicans in immunocompromised individuals.[3] It is important to successfully identify the particular species because of their specific resistance to antifungals acquired by them for effective treatment and management. Antifungal drugs, mainly azole groups such as fluconazole (FCZ), itraconazole (ICZ), ketoconazole (KCZ), are being used in treatment of initial and subsequent *Candida* infections. However, there have been difficulties in complete eradication of this fungus from patients owing to their resistance to azoles due to the genetic differences among the fungal species or overuse of azole drugs.[4]

In the present study, we have evaluated the prevalence of *Candida* colonizing in the oral cavity and estimated the colony-forming units (CFU/ml) counts in poorly controlled, moderately controlled, and well-controlled patients with diabetes. This study also aims to isolate and to identify the *Candida* species through culture method and their

Address for correspondence:
Dr. Lipsa Bhuyan,
Department of Oral Pathology and Microbiology, Kalinga Institute of Dental Sciences, Kalinga Institute of Industrial Technology Deemed to be University,
Bhubaneswar, Orissa.
E-mail: bhuyanlipsa@gmail.com

antifungal susceptibility in the study groups and correlate them with the control group.

Materials and Methods

Study comprised 200 individuals including 150 diagnosed cases of Type II DM and 50 healthy age- and sex-matched individuals who were randomly selected as control group. Demographic data as well as details such as fasting blood sugar level, postprandial blood sugar level, glycated hemoglobin level (HbA1c level), duration of the disease, and medication taken were recorded. The patients with diabetes were grouped into three groups according to their glycemic index: 50 well-controlled diabetes (HbA1c level ≤7%), 50 moderately-controlled diabetes (HbA1c range-7%–8%), and 50 poorly controlled diabetes (HbA1c level >8%). Participants having any known disease or condition that predispose to oral candidiasis, patients diagnosed with Type 1 DM, patients on antibiotics for 15 days before the sample collection, recent usage of corticosteroids, anemic patients, pregnant individuals, and previous history of treated mucosal diseases were excluded from the study. This study was approved by the Institutional Ethics Committee, Kalinga Institute of Dental Sciences, and informed consent was obtained from the participants participating in the study.

Microbial sampling

After a complete oral examination, unstimulated saliva samples were collected. A universal container containing 10 ml of sterile phosphate-buffered saline (PBS 0.1 M, pH 7.2) solution was supplied to each individual. They were asked to rinse the mouth thoroughly for 60 s. The oral rinse was then expelled into the sterile container. The samples were subjected to various mycological tests.

Microscopy, culture, and susceptibility

Each sample was examined microscopically before culturing in the KOH wet mount and Gram's stained smear was done to identify the budding yeast cells and pseudohyphae [Figure 1]. The material was inoculated in sabouraud dextrose agar medium and incubated at 37°C for 1-3 days. If no colonies were seen, it was considered negative. When positive for *Candida*, creamy white, smooth, and pasty colonies were observed [Figure 2]. Isolates of *C. albicans* were confirmed by germ tube test, and chlamydospore production was confirmed on corn-meal agar by the Dalmau plate technique [Figure 3]. Species with negative germ and negative chlamydospore were subjected to carbohydrate fermentation test and carbohydrate assimilation test for further identification of nonalbicans species. The isolates were also inoculated
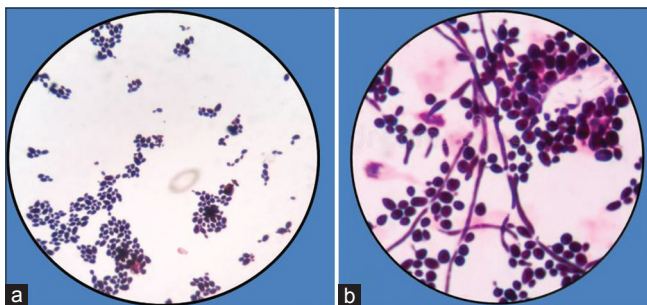

Figure 2: *Candida* **colonies on Sabouraud's Dextrose Agar showing creamy white, smooth, and pasty colonies**

Figure 1: (a) Smear from culture showing budding yeast cells. (b) Gram's staining of the smear showing budding yeast cells and pseudohyphae

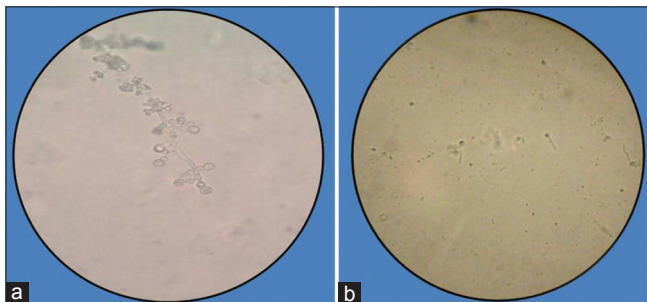**Figure 3: (a) Growth of** *Candida albicans* **on Corn-meal agar showing chlamydospores. (b) Germ tube production by** *Candida albicans*

Figure 4: Colored *Candida* **colonies on Chromagar media showing various species**
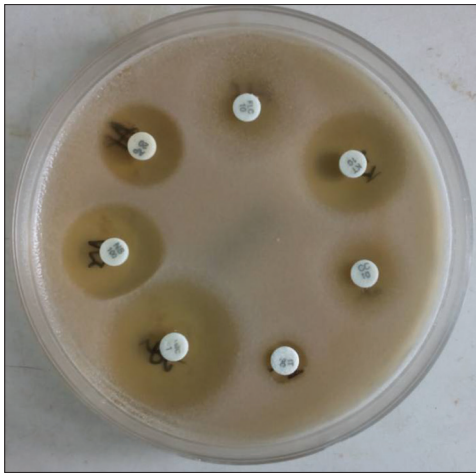
Figure 5: Antifungal susceptibility test by disk diffusion

Figure 6: Distribution of various *Candidal* species isolated from the oral cavities of well-controlled, moderately-controlled, poorly controlled diabetes, and control group

on CHROMagar *Candida* and incubated at 37°C in dark for 48 h. The colonies with color were considered for species identification [Figure 4]. Quantification of the colonies (number of CFUs/ml) was done by CFU/ml = 1000 × number of colonies/4. Antifungal susceptibility was evaluated by disk diffusion method with azoles and polyenes drugs [Figure 5].

Statistical analysis

The data collected were subjected to statistical analysis by Statistical Package for the Social Sciences software, version 22.0. Chi-square test was applied and $P \leq 0.05$ were considered significant.

Results

The sample population consisted of 200 patients with 118 males and 82 females. Their age varied from 42 years to 83 years with a mean age of 64.78 ± 8.02 years for poorly controlled diabetes, 61.48 ± 6.55 years for moderately controlled diabetes, 61.90 ± 6.91 years for well-controlled diabetes, and 64.78 ± 6.37 years for healthy controls. The glycated hemoglobin level varied from 5.5% to 14.1% (mean value = 7.82% ±1.65). There was a significant difference in frequency of *Candida* in poorly controlled diabetes when compared to moderately controlled diabetes, well-controlled diabetes, and normal patients ($P = 0.045$) [Table 1]. The higher number of colony count was seen among poorly controlled diabetes than well controlled, moderately controlled, and nondiabetic subjects [Table 2]. *C. albicans* was a prominent species in all the four groups. A comparatively low number of nonalbicans were seen in healthy individuals [Figure 6]. When antifungal sensitivity was done, *C. albicans* showed an increased resistance to FCZ in DM patients when compared to healthy controls ($P = 0.001$). A higher resistance of amphotericin B (AMB) (100%) and clotrimazole (CTZ) (50%–100%) against *Candida glabrata* was observed irrespective of the groups. However, KCZ, ICZ, voriconazole (VCZ),
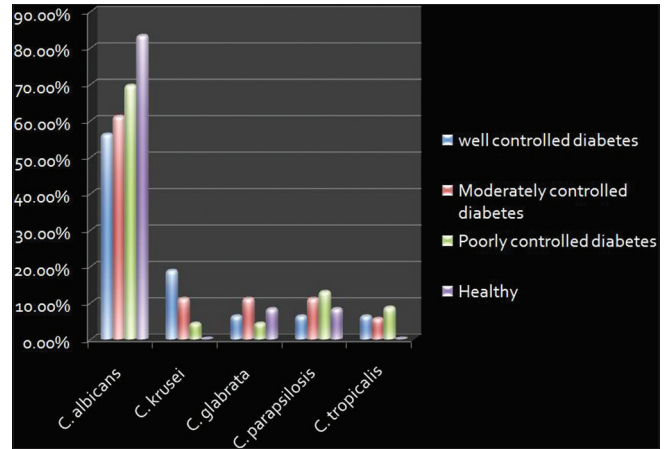
Table 1: Correlation between the groups and presence or absence of *Candida*

Presence of growth	Poorly controlled diabetes (%)	Moderately controlled diabetes (%)	Well-controlled diabetes (%)	Control (%)
Present	23 (46.0)	18 (36.0)	16 (32.0)	12 (24.0)
Absent	27 (54.0)	32 (64.0)	34 (68.0)	38 (76.0)
Total	50 (100.0)	50 (100.0)	50 (100.0)	50 (100.0)

5.554, *P*=0.045*

Table 2: Correlation between the groups and colony-forming units

CFU/ml counts	Poorly controlled diabetes (*n*=23), *n* (%)	Moderately controlled diabetes (*n*=18), *n* (%)	Well controlled diabetes (*n*=16), *n* (%)	Healthy control (*n*=12), *n* (%)	*P*
1-1000	0	0	4 (8.0)	2 (4.0)	0.017*
1001-5000	4 (8.0)	7 (14.0)	6 (12.0)	5 (10.0)	0.335#
5001-10,000	8 (16.0)	5 (10.0)	4 (8.0)	5 (10.0)	0.775#
>10,000	11 (22.0)	6 (12.0)	2 (4.0)	0	0.010*

CFU/ml: Colony forming units

and nystatin (NST) had good efficacy against the same. *Candida parapsilosis* showed resistance to ICZ (0%–14.13%). This fungi which were isolated from DM patients showed lower sensitivity to all other antifungal in comparison to those isolated from healthy individuals ($P = 0.01$). *Candida krusei* was resistant to VCZ but was well sensitive to FCZ and KCZ. *C. tropicalis* was resistant to AMB, intermediately sensitive (50%) to CTZ and KCZ but showed 100% sensitivity to FCZ, NST, and VCZ [Table 3].

Discussion

An increased frequency of infectious diseases and oral lesions in patients with DM is caused by the hyperglycemic environment that favors immune dysfunction which

Table 3: Comparison between antifungal susceptibility in diabetic and nondiabetic cases

Antifungal agent	Diabetic		Healthy		*P*
	S (%)	R (%)	S (%)	R (%)	
Amphotericin B					
C. albicans	10 (27.8)	26 (72.2)	4 (40)	6 (60)	0.242
C. glabrata	0	4 (100)	0	1 (100)	1.000
C. krusei	4 (66.67)	2 (33.33)	-	-	-
C. parapsilosis	4 (57.14)	3 (42.86)	1 (100)	0	0.010*
C. tropicalis	1 (25)	3 (75)	-	-	-
Clotrimazole					
C. albicans	27 (75)	9 (25)	5 (50)	5 (50)	0.242
C. glabrata	2 (50)	2 (50)	0	1 (100)	0.010*
C. krusei	2 (33.3)	4 (66.7)	-	-	-
C. parapsilosis	2 (28.6)	5 (71.4)	1 (100)	0	0.010*
C. tropicalis	2 (50)	2 (50)	-	-	-
Fluconazole					
C. albicans	3 (8.3)	33 (91.7)	10 (100)	0	0.001*
C. glabrata	4 (100)	0	1 (100)	0	1.000
C. krusei	5 (83.3)	1 (16.7)	-	-	-
C. parapsilosis	4 (57.14)	3 (42.86)	1 (100)	0	0.010*
C. tropicalis	4 (100)	0	-	-	-
Itraconazole					
C. albicans	9 (25)	27 (75)	5 (50)	5 (50)	0.242
C. glabrata	2 (50)	2 (50)	1 (100)	0	1.000
C. krusei	3 (50)	3 (50)	-	-	-
C. parapsilosis	1 (14.3)	6 (85.7)	0	1 (100)	0.012*
C. tropicalis	2 (50)	2 (50)	-	-	-
Ketoconazole					
C. albicans	32 (88.9)	4 (11.1)	6 (60)	4 (40)	0.055
C. glabrata	3 (75)	1 (25)	1 (100)	0	0.576
C. krusei	5 (83.3)	1 (16.7)	-	-	-
C. parapsilosis	3 (42.9)	4 (57.1)	1 (100)	0	0.010*
C. tropicalis	2 (50)	2 (50)	-	-	-
Nystatin					
C. albicans	25 (69.4)	11 (30.6)	9 (90)	1 (10)	0.235
C. glabrata	3 (75)	1 (25)	1 (100)	0	1.000
C. krusei	4 (66.7)	2 (33.3)	-	-	-
C. parapsilosis	6 (85.7)	1 (14.3)	1 (100)	0	0.010*
C. tropicalis	4 (100)	0	-	-	-
Voriconazole					
C. albicans	29 (80.6)	7 (19.4)	9 (90)	1 (10)	0.545
C. glabrata	3 (75)	1 (25)	1 (100)	0	0.576
C. krusei	2 (33.3)	4 (66.7)	-	-	-
C. parapsilosis	6 (85.7)	1 (14.3)	1 (100)	0	0.010*
C. tropicalis	4 (100)	0	-	-	-

C. albicans: Candida albicans; *C. glabrata*: Candida glabrata; *C. krusei*: Candida krusei; *C. parapsilosis*: Candida parapsilosis; *C. tropicalis*: Candida tropicalis; S: Sensitive; R: Resistant

potentially increases their morbimortality.[2] Among these oral lesions, oral candidiasis is most common lesion characterized by differential pattern of mucosal changes such as erythematous, pseudomembranous, and curd-like plaques. Although *Candida* is an oral commensal, its colonization increases in patients with diabetes. It is present

sub-clinically in absence of clinical lesion.[5] The various species diversity poses a challenge in management of such cases owing to its variable susceptible of the species to the antifungals.[4] An efficient identification of *Candida* species is a paramount to successful treatment and complete eradication. However, studies pertaining to *Candida* species resistance to antifungals in patients with diabetes are sparse. With this in view, the present study was conducted to isolate, identify, and compare the oral *Candida* species in diabetic and nondiabetic cases. This study also attempts to evaluate the susceptibility of the *Candida* species to commonly used antifungals.

DM is more common in developed countries with higher occurrence in ages between 45 and 64.[1] The age ranged from 48 years to 82 years in our study. Oral rinse technique was employed to collect the sample in our study. This method has been proved to be an appropriate and sensitive technique in comparison to other methods for accessing the overall yeast carriage.[6] The procedure involved in detection of the various species was by culture method in the present study which was proved as potent as polymerase chain reaction-restriction fragment length polymorphism in a study by Mohammadi *et al*.[3]

There was a higher prevalence of 38% for *Candida* carriage in patients with diabetes when compared to 24% in nondiabetic patients in our study which was consistent with the previous studies.[3,5,7-9] A much higher prevalence of 64% was reported by Belazi *et al*.[10] and 87% was reported by Premkumar *et al*.[11] This might reflect the distinguished ability of the fungus for epithelial cell adherence and genetic susceptibility of the patients with diabetes to infection. The diabetic group in the present study was categorized depending on the level of glycated hemoglobin concentration (HbA1c) since it denotes the average blood glucose level over a longer period that is over the past 3 months. A frequency of 24% was seen in healthy individuals in contrast to 46%, 36%, and 32% in poorly controlled, moderately controlled, and well-controlled diabetes, respectively. Thus, the rate of candidal carriage significantly correlated with the degree of glycemic control ($P = 0.045$) [Table 1] which was in consonance with study by Darwazeh *et al*.[12] Contradicting results were reported in few studies.[7,10,13] A direct correlation was observed between fasting blood sugar and candidal carriage by few studies.[14,15] However, Yar Ahmadi *et al*.,[16] Zomorodian *et al*.,[13] and Bremenkamp *et al*.[17] found no significant difference in *Candida* species frequency between diabetic group and healthy individuals. This contradiction may be attributed to small sample size, the geographic variation, difference in time of sampling, or use of alternative method.

The density of the harbored *Candida* was determined by calculating the CFU/mL. A CFU/mL >10000 was found in patients with diabetes but not in healthy patients [Table 2].

These findings were in consonance with other studies where higher mean CFU/mL was found in diabetic candidal carriers.[9,11,13,18] This rapid growth of yeast could be attributed to their increased salivary glucose level and altered oral microenvironment facilitating the growth of *Candida* in patients with diabetes. The presence of higher colonization of *Candida* for a longer duration should be considered as a potential risk factor for candidiasis in these patients.[13] However, few reports did not find any significant relationship.[17,19]

C. albicans was the predominant species in all the groups. The other species isolated in the diabetic group were *C. parapsilosis* (12.28%), followed by *C. krusei* (10.53%), *C. glabrata* (7.02%), and *C. tropicalis* (7.02%) [Figure 6]. *C. glabrata* (8.3%) and *C. parapsilosis* (8.3%) were the other species isolated other than *C. albicans* in healthy controls [Figure 6]. This was very much consistent with results of Martinez *et al*.[20] and Mohammadi *et al*.,[3] who moreover isolated *Candida kefyr* in healthy controls. Premkumar *et al*.[11] also detected *Candida dubliniensis* among other species. *Candida guillermondii*, *Candida lipolytica,* and *Candida lustitaniae* were isolated in Type II DM individuals by Patel *et al*.[21] No significant difference in the prevalence of various *Candida* was seen among the groups in this study similar to the previous reports.[13,16] A lower albicans to nonalbicans ratio was seen in the patients with diabetes [Figure 6]. This increase in the proportion of nonalbicans species may denote a change in oral environment such as reduced salivary flow due to salivary gland dysfunction and altered salivary composition.

Although the individuals in this study were free of clinical lesions, *in vitro* antifungal susceptibility to azoles (FCZ, VCZ, KCZ, CTZ, and ICZ) and polyenes (NST and AMB) were performed on all positive isolates by disk-diffusion method to study the susceptibility pattern and to determine if there is any emerging trend. Although nonsignificant, increased resistance to AMB against *C. albicans* was seen which was in disagreement to Yar Ahmadi *et al*.[16] and Zomorodian *et al*.[13] This may be due to building up of sterol intermediates and mutation of ERG3 in resistant strains.[11] Unlike results obtained by several authors[11,19,22] who obtained good sensitivity for FCZ against *C. albicans*, we observed poor *in vitro* activity against the yeast. Nearly 91.7% of patients with diabetes harboring the yeast were significantly resistant to FCZ in this study when compared to control group (*P* = 0.001) [Table 3]. This might be owing to regular prescription of these drugs in these individuals which might have resulted in a resistance against the drugs.

We found a higher resistance of AMB and CTZ against *C. glabrata* but good results against the fungi for KCZ, ICZ, and NST. A high efficacy of VCZ against the same was noted which was in agreement with the study by Lyon *et al*.[22] However, a few authors have reported a low efficacy of ICZ against *C. albicans* and *C. glabrata*.[22-24] *C. tropicalis*

was resistant to AMB, intermediately sensitive (50%) to CTZ, KCZ but showed 100% sensitivity to FCZ, NST, and VCZ. These reports were contradictory to the findings of Jiang *et al*.[25] *C. parapsilosis* was resistant to ICZ. These fungi when were isolated from DM patients showed lower sensitivity to all other antifungal in comparison to those isolated from healthy individuals (*P* = 0.01). *C. krusei* was resistant to VCZ but was well sensitive to FCZ and KCZ which was, in contrast, to study by Patel *et al*.[21] This species also had good efficacy against AMB, FCZ, KCZ, and NST but relatively resistant to CTZ and VCZ. The variability of drug resistance can be attributed to several factors such as degree of immunosuppression, prior exposure to a particular drug, changes in membrane lipid fluidity, and intrinsic resistance of *Candida* species.

Conclusion

There is disambiguity in microenvironment seen in the oral cavity of individuals with DM because of which they are more susceptible to opportunistic infections. A higher proportion of strains different from *C. albicans* species isolated from unstimulated saliva and their variable susceptibility to the commonly prescribed antifungals in diabetes indicate the necessity of the special mode of diagnostic and therapeutic management. This highlights the importance of routine candidal speciation and appropriate selection of prophylactic antifungal regimen after susceptibility testing, especially during intermittent visits. This might prevent systemic dissemination of this opportunistic yeast in such patients.

Failure of host defense in patients with diabetes necessitates an effective oral health regime. Dental health practitioners should make the patients aware of possible risk factors associated with poor oral health and should provide guidance for effective oral care. Better glycemic control, saliva replacement for dry mouths, maintenance of oral hygiene by the use of mouthwashes, toothbrushes and floss on a regular basis, and periodic visits to dentist may reduce the chances of oral candidal carriage and infections in individuals.

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Conflicts of interest

There are no conflicts of interest.

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