

Detection of species diversity in oral candida colonization and anti-fungal susceptibility among non-oral habit adult diabetic patients

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Abstract

Background and Objectives: Patients with diabetes mellitus are prone to secondary infections. In this study we aim to determine the prevalence of one such secondary infection (oral *Candida* colonization) and evaluate the influence of local and systemic factors on the oral candidal colonization in patients with diabetes mellitus. **Materials and Methods:** Forty non-insulin-dependent diabetic patients and 40 healthy individuals were included in this study. Samples were collected by using the oral rinse method. The candidal species were isolated and identified through phenotypic methods. An *in vitro* antifungal susceptibility profile was evaluated. Glycemic control, as determined by the glycosylated hemoglobin concentrations (HbA_{1c}) of the study subjects, was correlated with the candidal colonization. **Results:** Patients with diabetes showed a significantly higher prevalence of candidal colonization. The rate of carriage and density ($P = 0.001$) was higher. *Candida albicans* was the most predominantly isolated species, however, *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis* were also observed. Variable resistance toward the antifungal drugs (amphotericin B and fluconazole) was observed in the *Candida* isolated from diabetics, but not from healthy patients. Interestingly, a positive correlation was observed between glycemic control and candidal colonization. **Conclusion:** Diabetic patients had a higher candidal carriage rate, with a variety of candidal strains, which significantly varied in their resistance to routinely used anti-fungal agents. Interestingly the higher oral candidal colonization in diabetic patients is related to local and systemic factors, independent of their oral habits.

Key words: Antifungal susceptibility, candidal isolates, diabetic patients

INTRODUCTION

The *Candida* species, particularly *Candida albicans*, which are commensal in the human oral cavity, can become pathogenic and cause oral mucosal infections under immunocompromised or certain disease conditions.^[1] Patients with diabetic mellitus (DM) are predisposed to having an increased density of candidal growth in the oral cavity.^[2] Salivary dysfunction, reduced

salivary pH, and salivary hyperglycemia provide a potential substrate for fungal growth in these patients.^[3] Increased oral candidal colonization can predispose to rhomboid glossitis (central papillary atrophy), atrophic glossitis, denture stomatitis, pseudomembranous candidiasis (thrush), and angular cheilitis.^[4] However, these observations remain controversial, which may be due to some factors, such as, the age of the patients, duration of the disease process, pathophysiology of the disease, and to a certain extent racial and environmental differences.^[5]

The diabetic population is predicted to increase from 171 million in 2000 to 366 million by 2030.^[6] Almost three million deaths (5.2% of all deaths worldwide) per year are attributed to complications from diabetes,^[7] and a majority (~80%) of these patients are in developing

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countries.^[8] India is home to over 61 million diabetic patients – an increase from 50.8 million in 2010 – and it is expected to cross 100 million by 2030.^[9] In spite of these increased rates in the DM population of India, there is an apparent lack of published studies evaluating the prevalence and characteristics of oral fungal infections in these patients. Also the susceptibility of the *Candida* species to routinely used antifungal agents is not well investigated. Amphotericin B and fluconazole are the commonly used antifungal drugs for oral candidiasis.^[10]

The proportion of the non-albicans (NAC) species among the *Candida* species has been increasing over a few decades, due to variable reasons.^[11] The antifungal susceptibility varies significantly in contrast to *C. albicans*. Some NAC species are inherently less sensitive to fluconazole and may influence the development of resistance to fluconazole. Although *Candida albicans* is by far the most common cause of candidal infection, the incidence of candidiasis caused by other species, such as, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. dubliniensis* has also increased.^[12] These latter species tend to be less susceptible to commonly used antifungal agents such as fluconazole, and it has been suggested that this may account for their emergence as significant pathogens.^[13] Nevertheless, a reduced antifungal susceptibility in the non-albicans species and a correlation with routine fluconazole prophylactic use is suggested.^[14] Intrinsic and emerging resistance to azoles is a major challenge for therapeutic management and prophylactic strategies.^[15] Hence, there is a regular need for a meaningful antifungal susceptibility test for the oral *Candida* species. The classically used antifungal sensitivity tests (broth-based methodology (M-27A), CLSI methodology for molds, E-test agar-based testing methods, flow cytometry, and use of viability dyes) are time-consuming and labor-intensive; hence, a less time-consuming and more economic method such as the agar dilution method is described.^[16]

In this study we have evaluated the prevalence of the oral *Candida* carriage and the frequency of isolation of the *Candida* species (including its density and distribution) in the oral cavity. We have also studied the antifungal susceptibility of the *Candida* species, identified by determining the minimum inhibitory concentration (MIC) of amphotericin B and fluconazole, using the *in vitro* agar dilution method. The present study has further analyzed whether the significance of the different species and their densities can scale the immune status of diabetic patients. Our study emphasizes the importance of antifungal susceptibility testing of the *Candida* species from the oral cavity of adult diabetic patients.

MATERIALS AND METHODS

Clinical samples (oral rinse) were obtained from 80 subjects of both sexes (40 non-insulin-dependent diabetic patients (DG) attending a private diabetic center in Chennai, India, and 40 healthy controls (HC) who were age- and sex-matched volunteers (students and staff of Saveetha University) [Tables 1 and 2]. All the subjects voluntarily signed an informed consent form and the study was conducted after ethical clearance from the private hospital.

A structured questionnaire was developed for collecting information on demographics (age and gender), medical variables (diabetes type, duration, and presence of diabetes-related systemic diseases), and local factors (denture status, oral hygiene, and smoking).

The following were the inclusion criteria: (1) Diabetic patients who have been diagnosed at least three years back; (2) free of clinical manifestations of oral candidiasis. Individuals with a history of long-term antibiotic treatment, antiviral and antifungal treatment, xerostomia, anemia, HIV-related lesions, and any diseases related to oral candidal colonization were excluded from the study;^[17] (3) subjects with edentulous arches, denture wearers, and smokers were excluded from the study; (4) women subjects in the study had to be neither pregnant nor nursing.

The control group of 40 healthy individuals comprised of 23 females and 17 males, in the age range of 20 to 50 years. These individuals were volunteers from the dental staff, students, and laboratory assistants. None of the control subjects had oral symptoms and all presented with normal oral mucosa for the clinical examination. To assess the long-term glycemic control of the patients, blood samples from the diabetic patients were taken to measure the glycosylated hemoglobin concentrations (HbA_{1c}). For control subjects, the blood samples were used to measure the fasting plasma glucose level. According to the World Health Organization (WHO) definition of diabetes, those

Table 1: Criteria for susceptibility/resistance for the anti fungal drugs

Antifungal agents	Susceptible (µg/ml)	Resistance (µg/ml)
Amphotericin B	≤1	>1
Fluconazole	≤51.2	>51.2

Table 2: Distribution oacording to sex

Age	Diabetics (n=40) (%)	Controls (n=40) (%)	P value
<30	12.5	17.5	0.01
30-40	25	37.5	
40-50	62.5	45	

with values of 7 mmol/mL or higher were excluded from the study.^[18] Subjects who were enrolled for the study had undergone an oral examination primarily and an oral rinse was obtained from diabetic patients and healthy individuals.^[19]

Each subject was supplied with a sterile container containing 10 ml of phosphate-buffered saline (PBS; pH 7.2, 0.1 M) and he/she was requested to rinse the mouth thoroughly for 60 seconds and then return the rinse into the container. To reduce the effect of diurnal variation, meals, and tooth-brushing, the oral rinse samples were collected at the same time of the day (between 9 and 10 AM) and at least two hours after meals, drinking, or any oral-hygiene procedure. The material was centrifuged at 3500 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 1 mL of PBS. The resulting suspensions were 10-fold serially diluted in PBS, and 0.1 mL aliquots from appropriate dilutions, as well as from undiluted suspensions, were inoculated on Sabouraud's dextrose agar (SDA) supplemented with chloramphenicol (1 mg/ml) or onto chromagar candida plates. Both the SDA and the chromagar candida plates were incubated aerobically at 37°C for 48 hours. The candidal growth was identified to be round, smooth, creamy white colonies, and the total viable counts were performed on Sabouraud Dextrose Agar plates and expressed as colony forming units per one milliliter (CFU/ml). The candida species were identified based on the distinctive color of the colonies on the CHROMagar plates (Hi Media Pvt. Ltd.). Morphological and biochemical tests were performed to confirm the presumptively identified candidal species – the germ tube formation in serum and the carbohydrate fermentation test. An additional test for growth at 45°C was used to discriminate between *C. albicans* and *C. dubliniensis*.^[20]

Recovered isolates from the study group (DG) and control group (HC) were subjected to the antifungal susceptibility test by the agar dilution method.^[16] Various concentrations of antifungal drugs were prepared in compliance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. The antifungal drug, amphotericin B (0.0625-1 µg/ml) or fluconazole (0.2-51.2 µm/ml) was incorporated in doubling the dilutions in the Yeast nitrogen base agar medium. Ten microliters of suspension (10^6 Yeast cells/spores) were inoculated, incubated at 25°C, and at the end of 48 hours, the readings were recorded.

Preparation of stock solutions of antifungal agents

Amphotericin B (Hi Media, India)

The stock solution of amphotericin B was prepared by using 10 mg of amphotericin B dissolved in 1000 µl of Dimethylsulfoxide. The working solution was prepared by 1 in 1000 dilution.

Fluconazole (Cipla Pharmaceuticals Limited, India)

The concentration of the stock was 2 mg/ml. The stock solution was used directly without any further dilutions.

Culture medium

The medium Yeast nitrogen base (Hi Media, India) was dissolved in phosphate buffer pH 7 and autoclaved. Ten milliliters of the medium was dispensed in sterile bottles and the required concentration of amphotericin B and fluconazole (doubling dilutions) was incorporated into the medium. With each set a growth control without the antifungal agent was also included. The minimal inhibitory concentration (MIC) was determined by doubling the dilutions of antifungal agents for both drugs.

Preparation of the standard inoculum

The candidal strains were freshly subcultured onto SDA and incubated at 25°C for three days. The yeast cells and spores were suspended in sterile distilled water. Ten microliters of the standardized suspension was inoculated onto the control plates, and the media incorporated with the antifungal agents. The inoculated plates were incubated at 25°C for 48 hours. The readings were recorded at the end of 48 hours.

RESULTS

The minimal inhibitory concentration of the drug was determined, as the minimum concentration of the drug showed no growth when compared with that of the respective *Candida* species in the control plates.

The criteria for susceptibility/resistance of amphotericin B and fluconazole for the antifungal drugs are detailed in Table 1. The diabetic and control groups were homogenous in terms of age and sex [Tables 2 and 3]. However, the

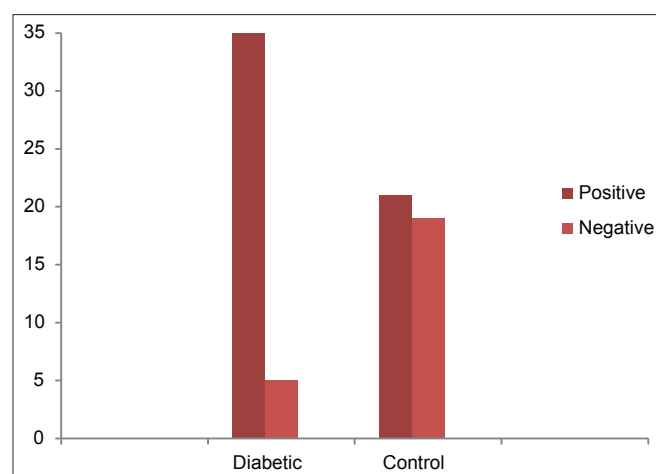


Figure 1: Candidal density among healthy controls and diabetic patients

control group showed a higher tooth brushing frequency than the diabetic group [Table 4]. The mean glycosylated hemoglobin level for diabetic patients was 8.75 (HbA_{1c}). From 40 asymptomatic diabetic patients, 35 (87.5%) candida isolates were recovered [Table 5 and Figure 1]. Thirteen (33%) and 18 (45%) *C. albicans* were isolated from diabetic patients and the control subjects, respectively. Interestingly, other non-albicans were also prevalent in the diabetic patients compared to control subjects [Figure 2]. Out of 35 culture-positive isolates, *C. dubliniensis* 14 (35%), *C. tropicalis* 3 (7.5%), and *C. parapsilosis* 5 (12.5%) were identified [Table 6].

The mean colony-forming units (CFU) per milliliter in the DG group (2600 CFU/ml) was significantly higher than in the HC group (690 CFU/ml).

The *in vitro* antifungal susceptibility revealed that the *Candida* isolated from diabetic patients had a variable resistance toward the antifungal drugs amphotericin B and fluconazole. Among the 35 species isolated from diabetic patients, a significant level of resistance to amphotericin B was observed in *C. dubliniensis* isolates (6/35 = 17%) compared to the other *Candida* species. Six out of 14 *C. dubliniensis* isolates were resistant to

the MIC of >1 µg/ml (17%), and eight isolates tested were found to be susceptible to the drug with an MIC range of 0.50-1 µg/ml. Thirteen *C. albicans* and one isolate of *C. tropicalis* were susceptible to amphotericin B with an MIC range of 0.50-1 µg/ml [Table 7].

Results from the susceptibility test for fluconazole revealed 27 sensitive isolates and eight resistant (8/35 = 23%, MIC >51.2 µg/ml) isolates. Of these, seven were *C. dubliniensis* and one was *C. tropicalis*. All the isolates of *C. albicans* and *C. parapsilosis* were susceptible to fluconazole with the MIC range between 25.6 and 51.2 µg/ml [Figure 3]. Antifungal-resistant *C. dubliniensis* and *C. tropicalis* were isolated from diabetic patients [Table 8 and Figure 4]. All the 21 isolates from 40 healthy controls showed susceptibility to both the drugs.

DISCUSSION

Increased rates of asymptomatic oral colonization with *Candida* are common in immunosuppressed patients.^[21] Due to the use of a variety of methods for yeast recovery from the oral cavity and quantification, studies on oral candidal colonization are contradictory; hence, to date the published literature regarding the relationship between

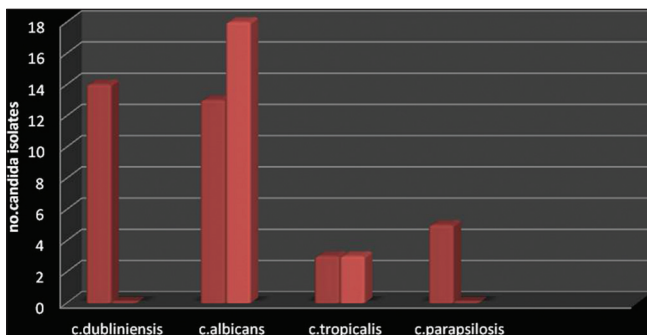


Figure 2: Candidal isolates among the groups

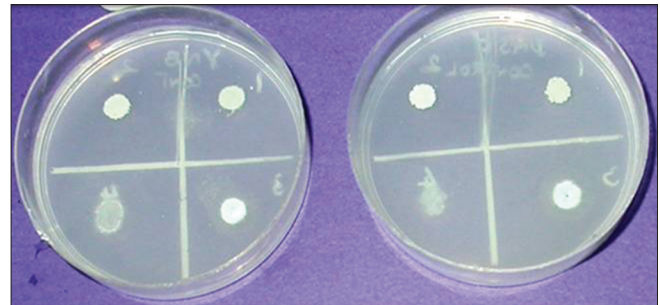


Figure 3: Antifungal-susceptible strains

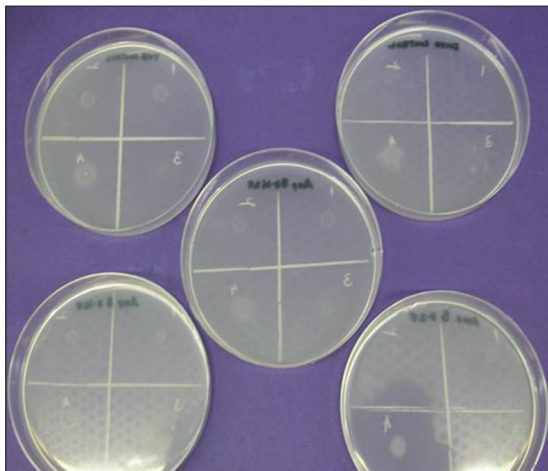


Figure 4: Antifungal-resistant strains

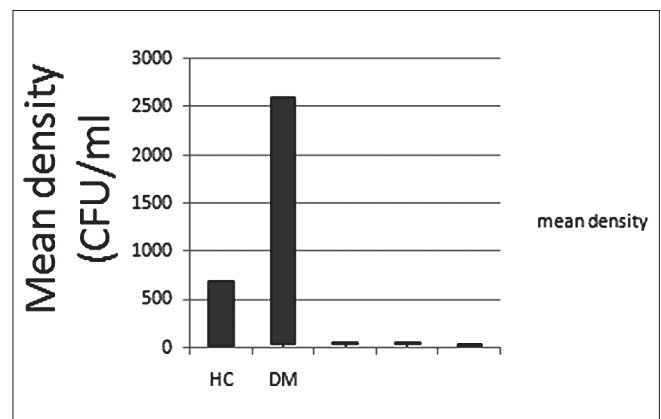


Figure 5: Comparison of mean density among healthy controls and diabetic patients

Table 3: Distribution according to sex

Sex	Diabetics (n=40) (%)	Controls (n=40) (%)	P value
Male	47.5	42.5	0.01
Female	52.5	57.5	

Table 4: Frequency of brushing habit

Brushing frequency	Diabetics (n=40) (%)	Controls (n=40) (%)	P value
Once a day	65	45	0.05
Twice daily	35	55	

Table 5: Candidal growth

Candidal carriage	Diabetic group (n=40)	Controls (n=40)
No. of candidal growth	35	21
% of candidal growth	88	53

Table 6: Candida species isolated from diabetics using Hichrome agar plates

Colony colour	Species	No. of isolates (%)
Blue green	<i>C. dubliniensis</i>	14 (35)
Green	<i>C. albicans</i>	13 (32.5)
Blue	<i>C. tropicalis</i>	3 (7.5)
Cream	<i>C. parapsilosis</i>	5 (12.5)

Table 7: Concentration of amphotericin B in µg/ml

Candida isolates	0.125	0.25	0.5	1.0	>1.0	Total
<i>C. albicans</i>	0	0	13	0	0	13
<i>C. tropicalis</i>	0	02	01	0	0	03
<i>C. dubliniensis</i>	0	0	08	0	06	14
<i>C. parapsilosis</i>	02	03	0	0	0	05

Minimum inhibitory concentration of amphotericin B on candidal isolates of diabetic patients

Table 8: Concentration of fluconazole in µg/ml

Candida isolates	6.4	12.8	25.6	51.2	>51.2	Total
<i>C. albicans</i>	0	0	06	07	0	13
<i>C. tropicalis</i>	0	0	0	02	01	03
<i>C. dubliniensis</i>	0	0	0	07	07	14
<i>C. parapsilosis</i>	0	0	05	0	0	05

Minimum inhibitory concentration of fluconazole on oral candidal isolates of diabetic patients

DM and candidal infection is debatable.^[22] The present study investigates, microbiologically, the prevalence of oral candidal colonization and evaluates the effect of some local and systemic factors that can potentially influence the candidal carriage rate and density in diabetic patients. The oral rinse technique used to collect samples in our study is an appropriate and sensitive technique for evaluating the overall yeast carriage when compared with other methods.^[19] The prevalence rate of the candidal species in diabetics was 87.5%, whereas, in the control subjects it was 50%, which is in concurrence with the previous

report.^[23] This higher incidence of *Candida* prevalence in DG may be explained by the fact that the normal oral flora is altered by the endocrine abnormalities in diabetes mellitus. The increased candidal colonization in diabetic patients may be attributed to the greater adherence of fungi to epithelial cells, facilitated by the increased glucose content in the saliva, genetic susceptibility to infection, altered cellular and humoral immune defense mechanisms, and local factors, including poor blood supply. However, contradicting our results is a recent study that shows no significant difference in the prevalence of *Candida* species between diabetics and healthy controls.^[24] A recent study conducted by Bremen Kamp *et al.*, has not found a statistically significant difference in the colonization of the *Candida* species between diabetics and healthy controls.^[24] This contradiction can be attributed to the fact that the glycemic levels have a positive correlation with the increased carriage rate and density in diabetic patients.^[3] This explains the reason for a higher carriage rate and density in the *Candida* isolates in the present study subjects, who had variable glucose levels in comparison to the healthy subjects.

The high number of CFUs in the DG group as compared to the HC group is consistent with the literature on immunocompromised patients.^[10] This is attributed to the alteration of the oral flora by the immunocompromised conditions. Candidal density seems to be a good predictor of the development of oral candidiasis in these groups of patients; however, some studies show no correlation between the density and clinical evidence of oral candidiasis.^[25] Although *Candida albicans* is the most frequently isolated species in our study, other species such as *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis* are also prevalent. This diversity in the *Candida* species is the most common finding in literature.^[26]

The intensive clinical use of antifungal agents has given rise to alarming cases of antifungal resistance.^[27] *In vitro* antifungal susceptibility has revealed that none of the *Candida* species from the control group are resistant to the two antifungal agents tested. However, isolates from the diabetic group have shown a significant level of resistance to antifungal drugs. None of the *C. dubliniensis* isolates, which have been resistant to fluconazole, have exhibited cross-resistance to amphotericin B, which is similar to the study by Moran.^[28] Replacement of *C. albicans* by *C. dubliniensis* is known to occur in patients treated with fluconazole. Oral microbial ecology is influenced by the antifungal pressure exerted by this drug, as these species are better able to adapt to the antifungal pressure persisting over those that are suppressed by the treatment.^[29] However, subjects included in this study have not been receiving any type of antifungal drugs for a period of at

least six months and the reason for this resistance may be due to the inherent resistance exerted by the *C. dubliniensis* isolates.^[28]

All the *C. albicans* isolates were susceptible to fluconazole and this was consistent with Manfredi *et al.*^[22] A recent *in vitro* study using the commercial Fungi Test kit revealed no difference in susceptibility between the candidal isolates from diabetics and non-diabetics to the six common antifungal agents tested.^[24] In comparison with the previous studies conducted in India, where they showed 7.8 and 21% of resistance toward fluconazole, the resistance against this conventional antifungal agent seemed to be on the higher side.^[12]

Our study shows a higher rate of resistance to conventional antifungal agents compared to other studies done in different countries.^[22] This could be explained by the misuse or overuse of the drugs by patients. This might lead to one of the serious consequences of the current medical practice in the country. The reduced azole susceptibility in the isolates of *C. dubliniensis* could be attributed to the overexpression of gene coding for CDR (Candida drug resistance) efflux pumps or the major facilitator (MDR1) superfamilies, which reduced the intracellular accumulation of fluconazole and increased the activity of the energy-dependent efflux mechanism.^[30] There was also an increased level of cytochrome P450 lanosterol demethylase, which reduced the activity of fluconazole. Increased expression of the *ERG11* gene encoding target enzyme, sterol 14 alpha demethylase (14DM), led to the alteration or overproduction of target enzymes, which lowered its affinity for flucoazole.^[31] It was also interesting to note the possible development of a drug-resistant *C. dubliniensis* phenotype among diabetic patients, which could be linked to the duplication of the chromosome carrying the *ERG11* gene encoding the antifungal drug target enzyme.^[32] Epidemiological switching and the inherent ability of *albicans* to generate a variety of phenotypes also played a major role in the resistance to antifungal drugs.^[33]

For example, *C. albicans*' transition to a hyphal growth facilitates its penetration into the tissue and enhances its virulence.^[34] Chau *et al.*, has described that mutations in *ERG3* evoke resistance to amphotericin B.^[35] Accumulation of the sterol intermediates in the resistant strain accounts for the decreased affinity of amphotericin B.^[36]

C. dubliniensis isolated from diabetes patients has shown a significant resistance to fluconazole and amphotericin B. Fluconazole resistance is a major problem faced by clinicians in the treatment of oral candidiasis, especially for immunocompromised patients. It is essential to perform an antifungal susceptibility test prior to initiating

any antifungal drug regimen. Moreover, the susceptible isolates of *C. dubliniensis* that were once susceptible may become resistant in future, which may have an implication in the change of antifungal drugs.^[28] Although our study includes a small sample size, nevertheless, it highlights a very important clinical problem of drug-resistant *Candida* prevalence among diabetic patients. However, the prime message that the disambiguity of the oral microenvironment of DM patients creates a conducive place compatible for the growth of non-*albicans* *Candida* species that are less susceptible to antifungal drugs, warrants clinician attention, to perform a sensitivity test before initiating therapy in addition to an accurate identification of the species.

CONCLUSION

Diabetic patients are more likely to carry species other than *C. albicans*, especially species like *C. dubliniensis*, which may not be sensitive to certain antifungal agents. Hence, culture and sensitivity testing will be of value for rationally selecting the appropriate antifungal drugs.

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