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Journal of Clinical Immunology and Microbiology

Research Article

Biofilm Formation, Virulence Factors and Antifungal Susceptibility of *Candida* spp. Isolated From the Oral Cavity of Diabetes Mellitus Patients

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Received Date: 29-11-2022; **Accepted Date:** 15-12-2022; **Published Date:** 23-12-2022

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Abstract

Background: *Candida* species colonize human microbiota and some conditions, such as immunosuppression or chronic illness, predispose the individual to fungal infections; among them, diabetes mellitus, a metabolic disorder frequently associated with higher rates of yeast infections.

Material and Methods: The prevalence of *Candida* species in the oral cavity of patients with diabetes mellitus was evaluated and the carriage was compared between type 1 and type 2 diabetic groups. In addition, in vitro susceptibility to antifungals, biofilm formation, cell surface hydrophobicity, and the production of hydrolytic enzymes were tested.

Santos JP | Volume 3; Issue 3 (2022) | JCIM-3(3)-060 | Research Article

Citation: Yamauchi LM, et al. Biofilm Formation, Virulence Factors and Antifungal Susceptibility of *Candida* spp. Isolated From the Oral Cavity of Diabetes Mellitus Patients. J Clin Immunol Microbiol. 2022;3(3):1-12.

DOI: http://dx.doi.org/10.46889/JCIM.2022.3306

Results: The results demonstrated the presence of different *Candida* species in the oral cavity of diabetic patients; and, also showed that type 1 diabetic patients are more susceptible to *Candida* colonization. Almost all isolates produce virulence factors such as proteases, phospholipases, or form biofilm; and they are sensitive to fluconazole and nystatin.

Conclusion: Colonization of *Candida* spp. oral isolates from type 1 and type 2 diabetic patients were similar; however, type 1 presented a higher colony-forming unit counting. Overall, *Candida* isolates from the oral cavity of diabetic patients are potential pathogens of candidiasis.

Keywords

Candida Species; Oral Isolates; Diabetes Mellitus; Antifungal Susceptibility; Virulence Factors

Abbreviations

DM: Diabetes Mellitus; T1DM: Type 1 Diabetes; T2DM: Type 2 Diabetes; PBS: Phosphate-Buffered Saline; SD: Sabouraud Dextrose; CFU: Colony-Forming Units; PCR: Polymerase Chain Reaction; MIC: Minimum Inhibitory Concentration; CLSI: Clinical and Laboratory Standards Institute; BSA: Bovine Serum Albumin, DZ: Degradation Zone; PZ: Precipitation Zone; CSH: Cell Surface Hydrophobicity Assay

Introduction

Worldwide, Diabetes Mellitus (DM) is recognized as one of the major health problems, mainly in low and middle-income countries and it is known as a disease and one of the key causes of other diseases. Autoimmune destruction of pancreatic beta cells causes Type 1 Diabetes (T1DM), and Type 2 Diabetes (T2DM) are triggered by resistance to insulin; both types result in almost 500 million adults living with diabetes worldwide [1]. These numbers are increasing every year, reaching 1/3 of the United States of America or the Canadian population, leading these patients to start insulin therapy [2]. DM is one of the largest risks to public health, boosting the susceptibility of patients to get infected, in addition, they also have a 2 folds risk of premature non-communicable diseases death [1].

DM patients, due to a deficiency in the functions of some immune response cells, are frequently colonized with *Candida* yeast [3]. *Candida* species are members of the human microbiota; however, they may become pathogenic during the impairment of the host immune response. Some conditions of DM patients, such as adhesion to epithelial cell surfaces, salivary flow and

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salivary glucose levels, facilitate *Candida* infection [4]. In addition to host factors, *Candida* yeasts can express virulence factors, such as hydrolytic enzymes and biofilm formation that contribute to the pathogenesis of candidiasis [5-8]. We address here, *Candida's* carriage of the oral cavity of diabetic patients seen at the primary healthcare unit in Maringá city, Paraná, Brazil, their characteristics, and the antifungal susceptibility.

Methodology

Ethics Statement and Study Population

The study was carried out on diabetic patients seen in 2011 at a primary healthcare unit in Maringá city, Paraná, Brazil. One hundred and twenty patients were enrolled and signed a written informed consent form to participate in this study, agreeing with the publication of this report and any accompanying images. The Ethics Committee approved the study protocol of Uningá (CEP no. 0017/11). A medical record of each patient was available, and a history of the previous diagnosis determined the diabetes type. According to the treatment they were taking for diabetes, they were subdivided into Type 1 Diabetes Mellitus (T1DM) included those patients who were on insulin and Type 2 Diabetes Mellitus (T2DM) included those patients who were on oral medication and diet control.

Sample Collection and Identification

Samples were collected using the oral rinse method according to Samaranayake et al. [9], with some modifications. Briefly, patients rinsed the oral cavity using 10 mL of sterile physiological solution (NaCl 0,85%) for 30 seconds and spit it out into a clean container. Samples were centrifuged at 1,500 x g for 10 min at 4 ºC, supernatants were discarded, and the pellets were resuspended in 0.5 mL of 50 mM Phosphate-Buffered Saline (PBS), pH 7.4 containing 0.15 M NaCl. 100 µL sample was spread on Sabouraud Dextrose (SD) agar (Himedia, India) supplemented with 0.1 mg/mL chloramphenicol (Inlab, Brazil) and in CHROMagar *Candida* medium (Himedia, India). The cultures were incubated at 37 ºC for at least 48 h and the colonies were counted and expressed as the number of Colony-Forming Units (CFU). The presence of *Candida* yeasts was confirmed by Gram staining and pigmentation characteristics on chromogenic agar. Each isolate was identified at the species level by standard mycological methods [10]. Species identification was also confirmed by molecular identification methods based on PCR [11]. All yeasts were maintained at -80 ºC in SD broth containing 30 % glycerol.

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Antifungal Susceptibility Testing

The Minimum Inhibitory Concentration (MIC) of fluconazole (Sigma) and nystatin (Sigma) for all isolates was determined by the broth microdilution assay for yeasts according to the Clinical and Laboratory Standards Institute guidelines, using M27-S4 recommendations [12]. *Candida parapsilosis* ATCC 22019 was included in each assay as quality control. The results were presented as a range of variations of minimum and maximum minimum inhibitory concentration values obtained for all *Candida* species. The antifungal concentration tested ranged from 128 to 0.25 µg/mL for fluconazole and 64 to 0.125 µg/mL for nystatin. Two wells were also included as growth and sterility controls. Interpretative criteria for susceptibility to fluconazole were those published in the CLSI [12]. For nystatin, MIC was defined as the value in which 100% growth inhibition was observed [13].

Determination of Protease and Phospholipase Activities

Candida isolates were assayed on minimal medium (55 µmol/L glucose, 11 mol/L KH_2PO_4, 6 mol/l KCl, 2 mol/L $MgSO_4.7H_2O$, 65 mol/L $FeSO_4$, 61 mol/L $ZnSO_4$) supplemented with 0.5% Bovine Serum Albumin (BSA, Sigma) and SD agar plates containing 4.0% egg yolk as protease and phospholipase substrate, respectively. To determine the protease activity [14], the isolates were previously cultured at 37 ºC for 18 h in minimal medium broth supplemented with 0.5% BSA, pH 4.0, to induce enzyme secretion. To determine phospholipase activity, the cell suspensions were obtained from a 24 h SD broth yeast culture, and the assay was carried out in SD agar supplemented with 4% egg yolk, 350 µmol/L NaCl, and 6.5 µmol/L $CaCl_2$, pH 4.5. For both assays, cells were counted in a hemocytometer, and a 10 µL suspension of $1x10^6$ yeasts was spotted on the surface of the agar medium. Cultures were incubated at 37 ºC for 96 h, afterwards, the diameter of the degradation (protease activity - Dz) or precipitation (phospholipase activity - Pz) zone around the colony was determined. The enzyme activity was determined by the ratio between colony diameter and colony diameter plus the degradation/precipitation zone. The isolates were classified according to Price, et al., where Dz/Pz values of 1 indicate no detectable protease or phospholipase activity, respectively. All *Candida* isolates were tested in triplicate and the experiment was carried out on three independent experiments [15].

Cell Surface Hydrophobicity Assay

The biphasic aqueous-hydrocarbon assay was used to assess Cell Surface Hydrophobicity Assay (CSH) on *Candida* isolates [16]. Briefly, all isolates were grown at 37 ºC for 24 h in SD broth. Yeasts were centrifuged, washed twice, and the cell densities were adjusted to an absorbance of 0.4 at 660 nm in 5 mL of PBS. A volume of 2.5 mL of cell suspension was added

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to two sterile glass test tubes, in addition, 0.5 mL of xylene (Merck) was added to one tube. After 10 min incubation in the water bath at 37 ºC, the test tube was vigorously mixed for 1 min and incubated for an additional 30 min under the same conditions. The aqueous phase was carefully removed, and the absorbance was measured at 520 nm. CSH was expressed as the percentage decrease in the optical density of the test compared with the control. Thus, the more significant the change in absorbance of the aqueous phase, the more hydrophobic the yeast sample. Each assay was performed on two separate occasions with duplicate determinations each time.

Biofilm Formation

Biofilm production was determined on polystyrene flat-bottomed 96-well microtiter plates (Techno Plastic Products, Switzerland) according to Shin, et al., with modifications [17]. Briefly, *Candida* isolate was grown at 37ºC for 24 h in SD broth and a 20 µL SD-suspension of 6 x 105 yeasts was placed in each well containing 180 µL SD broth. The plates were incubated at 37ºC for 24 h and washed once with sterile distilled water. Crystal violet staining was used to quantify bulk biofilm production [18]. Briefly, after biofilm formation, each well was washed twice with PBS and the plate was dried for 20 min at 35 °C. Biofilms were stained with 110 µL of 0.4% aqueous crystal violet solution for 45 min, washed three times with ultra-pure sterile water, and discolored with 200 µL of 95% ethanol. After 45 min, 100 µL of distaining solution from each sample was transferred to a new plate and measured with a spectrophotometer plate reader (Universal Microplate Reader ELx 800; Bio-Tek Instruments, USA) at 595 nm. Samples exhibiting a very intense blue color were diluted 1:4 with sterile water before performing a second absorbance reading. The absorbance values of the negative controls (containing no cells) were subtracted from the values of the test wells to minimize background interference. Experiments were carried out in triplicate on two different occasions.

Statistical Analysis

Demographic data were reported as mean values or percentages of the total. The differences between group rates were assessed by Student's t-test, $p<0.05$ was considered statistically significant. All statistical analyses were performed with the software Graphpad prism 4.0 for windows (Prism Graphpad software, San Diego CA, USA).

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Results and Discussion

T1DM Patients Present an Increased Counting of *Candida albicans* in Oral Yeast Carriage Analyses

One hundred and twenty patients were enrolled and signed a written informed consent form to participate in this study. The subjects consisted of 49 males (34.2%) and 79 females (65.8%), and most of them were elderly; the age mean was 67.8±13.9 years (ranging from 5 to 93 years). A medical record of each patient was available, and a history of the previous diagnosis determined the diabetes category: T1DM included those patients who were on insulin treatment and T2DM included those patients who were on oral medication and diet control. Among them, 35% were T1DM patients and 65% were T2DM patients, with no differences between their ages mean, 68.3±10.2 and 65.5±19.1, respectively.

Identification and Characterization of *Candida* spp Isolated in Oral Yeast Carriage Analyses

A total of 66 patients were positive for *Candida* carriage (Table 1). The T1DM group presented a higher *Candida* carriage than T2DM patients, 32 and 34 individuals, respectively (Table 1). The colony counts ranged from 5 to up 103 CFU (Fig. 1), and interestingly, T1MD patients had a significant increase in CFU compared to T2DM patients ($p<0.05$).

All cases (N = 120)	**T1DM (*n* = 42), *n* (%)**	**T2DM (*n* = 78), *n* (%)**
Yeast Culture		
Negative (*n* = 54), *n*	10 (23.8)	44 (56.4)
Positive (*n* = 66), *n*	32 (76.2)	34 (43.6)
Species		
C. albicans (*n* = 42), *n*	23 (71.9)	19 (55.9)
C. tropicalis (*n* = 11), *n*	5 (15.7)	6 (17.6)
C. glabrata (*n* = 9), *n*	3 (9.3)	6 (17.6)
C. dubliniensis (*n* = 3), *n*	1(3.1)	2 (5.9)
C. parapsilosis (*n* = 1), *n*	0	1 (3.0)
Legend: N= total number of cases, n= number of diabetes-type patients or yeast culture or yeast species.		

Table 1: Distribution type of diabetes and frequency of yeast culture and species found in the saliva of diabetic patients.

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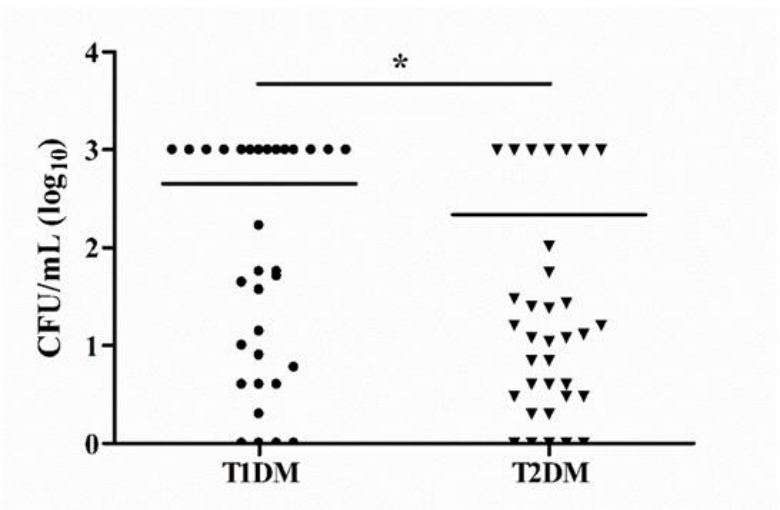

Figure 1: *Candida* colony counting from samples of oral cavities of diabetes Mellitus type 1 and 2 patients (n = 66 patients). *$P < 0.05$, for a comparison between T1DM and T2DM.

Each *Candida* isolate was identified at the species level (Table 1), in both groups, *C. albicans* represented the most frequently isolated species followed by *C. tropicalis, C. glabrata, C. dubliniensis* and *C. parapsilosis* (Table 1). The distribution of *Candida* species is close to the Brazilian epidemiology, with a predominance of *C. albicans, C. tropicalis* and followed by others [19].

Candida ability to produce hydrolytic enzymes used in the invasion of host tissue and liberation of nutrients is related to its overgrowth [20]. The enzyme activity was determined by the ratio between colony diameter and colony diameter plus the degradation/precipitation zone [15]. The results showed that some *Candida* isolates presented protease or phospholipase activities (Table 2). These activities have seemed in a lower percentage of samples [21, 22].

Number of Isolates					
	C. albicans	*C. tropicalis*	*C. glabrata*	*C. dubliniensis*	*C. parapsilosis*
Scoring of Protease Activity[a]					
Negative	37	8	5	3	1
1 +	-	-	-	-	-
2 +	5	3	4	-	-
3 +	-	-	-	-	-

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Scoring of Phospholipase Activity[a]					
Negative	36	8	4	3	1
1 +	-	1	3	-	-
2 +	6	2	2	-	-
3 +	-	-	-	-	-
Cell Surface Hydrophobicity[b]					
≤ 30%	19	5	3	1	-
30 - 70%	15	3	-	-	1
≥ 70%	8	3	6	2	-
Biofilm Formation[c]					
≤ 0.100	3	1	2	1	-
0.101 - 0.250	7	2	2	-	-
0.251 - 0.500	7	2	3	1	1
≥ 0.501	25	6	2	1	-
Fluconazole[d]					
Range of MIC (µg/mL)	0.125 - 32	0.125 - 2	0.125 - 8	0.125	0.125
Resistant	1 (2.4)	0	0	0	0
Susceptible	40 (95.2)	12 (100)	2 (22.2)	3 (100)	1 (100)
Susceptible dose-dependent	1 (2.4)	0	7 (77.8)	0	0
Nystatin[e]					
Range of MIC (µg/mL)	0.125 - 2	0.125 - 1	0.125 - 1	0.125 - 0.5	0.5
Resistant	0	0	0	0	0
Susceptible	42 (100)	12 (100)	9 (100)	3 (100)	1 (100)
Susceptible dose-dependent	0	0	0	0	0

Legend: The interpretative criteria were: aThe protease and phospholipase activities were determined by calculating the ratio between colony diameter and colony diameter plus degradation/precipitation zone (Dz/Pz) as previously described [15]. The enzymes activities were scored into four categories: Negative, Dz/Pz of 1.0; low activity (1+) for 0.64 < Dz/Pz < 1.0; intermediate activity (2+) for 0.30 < Dz/Pz ≤ 0.64; high activity (3+) for Dz/Pz ≤ 0.30. bCell surface hydrophobicity was scored as either weakly (<30%) or moderately (30%-70%) or strongly (70%)

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hydrophobic as previously described[16]. cThe adherent biofilm layer was scored as either negative (≤ 100) or weakly (0.101 a 0.250), moderately (0.250 a 0.500), or strongly (>0.500) positive as previously described [18]. The interpretative criteria for susceptibility to fluconazole and nystatin were those published [13] and Paula et al.[22], respectively dFluconazole: MIC ≤ 2 µg/mL, susceptible; MIC = 4 µg/mL, susceptible dose-dependent); MIC ≥ 8 µg/mL, resistant. eNystatin: MIC ≤ 4 µg/mL, susceptible; MIC = 8-32 µg/mL, susceptible dose-dependent; MIC > 64 µg/mL, resistant. n= number of yeast species.

Table 2: Protease, phospholipase activities, cell surface hydrophobicity, biofilm formation, and *in-vitro* susceptibility to fluconazole and nystatin of *Candida* isolates from diabetic patients.

To colonize and cause disease, *Candida* species must evade the host immune response and multiply, therefore, yeast adhesion on host surfaces is essential for the establishment of colonization. This mechanism involves many biological factors such as the presence of adhesins and cell surface hydrophobicity resulting in invasion and biofilm formation, and this is strongly related to growth on tissues or implanted medical devices [4]. Cell Surface Hydrophobicity assay (CSH) on *Candida* isolates was assessed using a biphasic aqueous-hydrocarbon test [16]. The mean relative CSH of all isolates was 47.40±26.58, ranging from 1.16 to 93.50 (Table 2), these data showed that *Candida* isolates have the potential of yeast adhesion to epithelial cells and extracellular matrix proteins increasing the potential to form biofilm.

Biofilm production was determined on polystyrene flat-bottomed 96-well microtiter plates [22]. Our results showed that the majority of *Candida* isolates formed a biofilm (Table 2). The main concern is that *C. albicans* is the leading fungal microorganism found in implanted medical devices and biofilm increases resistance to antifungal drugs, which makes it a challenge and leads to many clinical complications [23,24].

Finally, the Minimum Inhibitory Concentration (MIC) of fluconazole and nystatin for all isolates was determined by the broth microdilution assay for yeasts. Most *Candida* isolates were susceptible to fluconazole (MIC ranging from 0.125-32 µg/mL), 8 isolates were susceptible dose-dependent and one C. *albicans* isolate was resistant to fluconazole (Table 2). Regarding nystatin (Table 2), all isolates were susceptible (MIC ranging from 0.125 to 2 µg/mL).

Overall, *Candida* isolates from oral cavities of diabetes patients are potentially pathogenic, capable of forming a biofilm. T1DM patients are more susceptible to *Candida* colonization and indeed, they presented higher CFU counting. A study of the oropharyngeal microbiome of elderly individuals showed several microorganisms such as bacteria and yeast that can become opportunistic pathogens in these individuals with debilitated immune response or health problems; its colonization appears to be associated with some physiological changes as salivary pH and loss of glycemic control [3]. Our study found that C. *albicans* is the most common species of the oral cavity of diabetes patients, followed by *C. tropicalis, C. glabrata, C.*

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dubliniensis and *C. parapsilosis* similar to another study [25]. An increase in the frequency of different *Candida* species isolation has clinical importance since they may have a reduced susceptibility to antifungals compared to *C. albicans*, although most *Candida* isolates were susceptible to antifungals tested [26]. Fluconazole was established as one of the main drugs to treat most cases of candidiasis, in addition, it is the choice of initial treatment for suspected fungal infections. The main concern is that although most isolates showed susceptibility to fluconazole and nystatin *in-vitro*, we detected a reduced susceptibility in some isolates, especially in reading after 24 hours. Monitoring antifungal resistance among *Candida* species will help in empirical treatment due to the knowledge of what is happening in the community, especially among diabetes patients.

Conclusion

Type 1 and type 2 diabetic patients present similar rates of *Candida* spp. oral carriage colonization; however, type 1 presented a higher colony-forming unit counting. These isolates are potential pathogens of candidiasis, presenting several virulence factors to enhance *Candida* pathogenesis, such as phospholipase and protease activity. In addition, they are capable to form biofilm, and some isolates present fluconazole resistance.

Acknowledgment

The authors thank the technician Ediel C. Costa for his help with laboratory maintenance. Natalia de S. Botelho, Alexandre T. Morey, Eliandro R. Tavares, received fellowships from Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES, Finance Code 001). Sueli F. Yamada-Ogatta and Lucy M. Yamauchi received PQ fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Ethical Approval

This study was previously approved by the Ethics Committee of Uningá (CEP no. 0017/11). All procedures performed in studies involving human participants were following the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest

The authors declare that have no competing interest and not any conflict of interest.

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Santos JP | Volume 3; Issue 3 (2022) | JCIM-3(3)-060 | Research Article

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