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ABSTRACT

Objective: To determine number, species of *Candida* and *Candida* resistance to antifungal therapy according to the metabolic control state and the associated salivary changes in patients with type 2 diabetes mellitus (DM2).

Materials and methods: Samples of non-stimulated saliva were collected from 52 patients with DM2. Salivary pH was measured and cultured on Sabouraud glucose agar and the values of CFU/ml were calculated. The species were presumptively identified using CHROMagar *Candida*® plates, and identification was confirmed by polymerase chain reaction (PCR). *C. albicans* isolates were cultured on SGA tetracycline agar with nystatin and fluconazole diffusion disks to measure susceptibility.

Results: Sixty six percent of the yeasts isolated were *Candida albicans*, followed by *C. glabrata* (20.7%). In patients with decompensated DM2, there was an inverse association between HbA1c value and salivary pH. At higher levels of salivary acidification, a greater diversity and quantity of yeasts of the genus *Candida* were observed. With nystatin, higher inhibition was observed at lower pH.

Conclusions: The antifungal therapies could be more effective if it consider, qualitative salivary characteristics as pH, that could determine the susceptibility of species of Candida to at least to nystatin, which is the most used antifungal for treatment to oral candidiasis in patients with DM2.

Introduction

Type 2 diabetes mellitus (DM2) is the most common metabolic disease in the world. It affects approximately 10% of the population, and the global projection is that diabetes will affect 50% of the population in the year 2030 [1]. It is generally acknowledged that patients with diabetes mellitus are more susceptible to fungal infections, particularly with *Candida albicans* (*C. albicans*). Studies have shown that patients with diabetes mellitus have an increased frequency of oral *Candida* carriage and increased risk of candidiasis, which is related to poor metabolic control, neutrophil dysfunction, reduced salivary flow, high glucose concentrations in the blood and saliva and a deficient immune response [2]. The best known method to evaluate metabolic control of DM2 is the measuring of glycosylated haemoglobin (HbA1c), which evaluates the percentage of haemoglobin to which glucose is bound over a period of 60–90 days [3]. The goal of treatment of type 2 diabetes is to maintain the level of HbA1c below 7%, which is considered the level of metabolic compensation. Above this level, affected individuals experience complications associated with the disease progression and are more susceptible to developing infections, especially candidiasis [4].

In addition to exhibiting changes in the immune response associated with the progression of DM2, DM2 patients with metabolic decompensation (HbA1c > 7%) exhibit changes in the composition and quantity of saliva, such as greater acidification [5]. This phenomenon might be due to the increased presence of glucose in the saliva in DM2, which favours the growth, activity and resistance of opportunistic microorganisms, especially fungi of the genus *Candida* [6,7]. *C. albicans* is the most commonly identified species of *Candida* in saliva and is the main cause of oral candidiasis in DM2. Higher levels of *C. albicans* and a greater presence of non-*albicans Candida* species have been reported in patients with decompensated DM2 [8–10]. In addition, an acid salivary environment could potentiate pathogenicity factors such as a more invasive morphology, especially in *C. albicans*, thereby increasing resistance to antifungal therapy [10]. Current therapies for oral candidiasis in DM2 patients include the administration of topical antifungal agents such as nystatin or some azoles, including fluconazole [11]. There is some evidence that *Candida* species, particularly *C. albicans*, can develop resistance to antifungal agents and decrease the plasma availability of fluconazole [12]. However, information regarding *Candida* diversity in DM2 and its resistance to

antifungal agents commonly used in oral candidiasis remains scarce.

Poor glycaemic control is associated with higher prevalence rates of *Candida* carriage and higher densities in diabetic patients. In addition, a high prevalence of *C. dubliniensis* in diabetic patients was found, and this species might be misdiagnosed as its morphologically similar relative, *C. albicans* [8]. As DM2 is a high-frequency disease and the majority of affected patients are decompensated [13], oral candidiasis is a common consequence, and antifungal resistance is a global health problem. Protocols to treat these complications according to the different states of metabolic control and possible associated salivary changes are lacking. Therefore, the objectives of this study are to quantify the number and species of *Candida* according to metabolic control state and evaluate the resistance of these species in relation to salivary pH. The results of this study can be used to improve therapies to treat oral candidiasis in DM2, potentially providing greater effectiveness and fewer adverse effects.

Materials and methods

Subjects

Salivary samples were collected from 52 patients diagnosed with DM2 from the Chilean Diabetes Association (Association of Diabetes of Chile, ADICH). The patients had signed a consent form approved by Ethics and Biosafety Committee of Faculty of Dentistry, University of Chile (Number 2013/01), respecting the principles of the Declaration of Helsinki for research with human beings. Glycated haemoglobin (HbA1c) results were requested within no more than two days before salivary sampling. All patients were being treated pharmacologically for diabetes. Exclusion criteria included pregnancy, neurological damage, rheumatoid disease, neck and head irradiation, acute inflammatory processes in the mouth and the use of oral removable prosthesis or orthodontic appliances.

Sample collection

All samples of non-stimulated saliva were taken by a single operator between 10 and 12 am. Following a distilled water mouthwash and five minutes under relaxed conditions, patients deposited saliva for five minutes in 50 ml centrifuge tubes (Falcon, BD, Austin, TX) according to the protocol described by Navazesh et al. [14]. The samples were stored at 5 °C and transported maximum 15 minutes after the collection to the Laboratory where they were processed.

Salivary pH determination

Sample pH was measured immediately after the collection at room temperature with a digital pH meter that had been previously calibrated for 15 seconds (PL-600 EXDO-OMEGA, ISO-9001). Patients were grouped by pH ranges (6.5–7; 7.01–7.5; 7.51–8; >8).

Yeast culture and presumptive identification

The tubes containing the saliva samples were vortexed for 15 s. Then, for each sample, 100 µl of saliva was collected and placed on a petri dish containing Sabouraud glucose agar (SGA) (tetracycline 50 µg/ml). The plates were incubated for 48 h at 30 °C and the colony forming units (CFUs) per millilitres of saliva (CFU/ml) were estimated. An aliquot of 100 µl of sample was used for presumptive identification by using CHROMagar *Candida*® plates according to the manufacturer's instructions.

DNA extraction

A colony from isolated strains cultured in agar plates was used to prepare a standardized cell suspension in sterile NaCl 0.9% (2 absorbance units, 560 nm). For DNA extraction, an aliquot of 125 µl from this suspension was placed on filter paper and left to dry at 37 °C for 20 minutes. Then, an additional aliquot of 125 µl of each strain was applied to the disk. After drying, the filter papers were cut, obtaining a punch for each isolate, and then washed with 200 µl NaOH 20 mM for 30 minutes and with 200 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8,0) for five minutes.

DNA amplification

For those samples in which presumptive identification was ambiguous, we distinguished *C. albicans* and *C. dubliniensis* by using polymerase chain reaction (PCR) amplifying primers that are specific for cell wall protein (HWP-1). This technique [15] produces two DNA fragments that can be differentiated by size: 930 bp segments for *C. dubliniensis* and 1.180 bp segments for *C. albicans*. The primers were Wall F: (5′-GTTTTTGCAACTTCTCTTTGTA-3′) and Wall R: (5′-ACAGTTGTAT CATGTTCAGT-3′). To confirm the presumptive identification of *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei* and *C. guilliermondii*, we amplified primers for internal transcribed spacer regions for fungal ribosomal DNA ITS1 and ITS4: ITS-1: (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4: (5′-TCCTCCGCT TATTGATATGC-3′). The PCR mix consisted of 1.5 µl 10× PCR buffer, 0.6 µl 50 mM $MgCl_2$, 0.3 µl 10 mM dNTPs, 3 µl 5 M betaine, 0.3 µl 5 U/µl Taq polymerase, 0.6 µl 25 µM of each primer, distilled water and gDNA (punch). For WF and WR, amplification was performed as follows: denaturation at 95 °C for three minutes, followed by 35 cycles of denaturation at 95 °C for one minute, primer annealing at 50 °C for one minute and extension at 72 °C for two minutes, followed by a final extension at 72 °C for 10 minutes. For ITS-1 and ITS-4, amplification was performed as follows: denaturation at 95 °C for five minutes, followed by 34 cycles of denaturation at 94 °C for 45 s, primer annealing at 50 °C for one minute and extension at 72 °C for 10 min, followed by a final extension at 72 °C for 10 minutes.

Agarose gel electrophoresis

Amplified products were separated by horizontal electrophoresis in 2% agarose gel. Gels were stained by incubation with ethidium bromide (0.5 µg/ml in water). The ethidium bromide-stained gels were visualized using UV transillumination. As positive controls, we used *C. albicans* ATCC 90029, *C. dubliniensis* CD36, *C. glabrata* ATCC 2001 and *C. tropicalis* ATCC 750, and DNA ladders of 50 bp and 100 bp were used as reference.

Determination of susceptibility to antifungal agents

The CLSI M44-A (Espinel-Ingroff, 2007) method was used to test the susceptibility of different strains of *C. albicans* to fluconazole therapy and nystatin *in vitro*. After cultivation in CHROMagar *Candida*® for 24 h at 35 °C, a standardized inoculum of 0.5 McFarland suspension was prepared in 0.15 M of sterile sodium chloride. For diffusion assay, 100 mm petri dishes were used with 20 ml of SGA supplemented with chloramphenicol. The inoculum was spread on the surface of each petri dish in three directions and allowed to dry for 10–15 minutes at 35 °C. Subsequently, a disc of fluconazole (OXOID®) or nystatin (OXOID®) 6 mm was placed equidistant from the plate margins. After incubation for 24 h at 35 °C, the plates were examined on a black, non-reflective surface illuminated by reflected light. The diameters around the disks were measured from the nearest millimetre to the point where prominent reduction in growth was observed. According to the diameter of the inhibition zone, the species were classified as sensitive (more than 20 mm), intermediate (between 12 and 19 mm) or resistant (less than 11 mm).

Statistical analysis

Fisher's exact test was used to compare between compensated and uncompensated patients, gender variables, metabolic control and yeast carriage. The Kruskal–Wallis test was used to compare species diversity, and Student's *t*-test was used to compare susceptibility to treatment with fluconazole/nystatin. A *p* value <.05 was considered statistically significant. Analysis was performed by using the software STATA® 9.0.

Results

Subject characteristics

Of the 52 subjects diagnosed with DM2 that were recruited for this study (26 with HbA1c > 7% and 26 with HbA1c < 7%), 40 (76.9%) were women and 12 (23.1%) were men. The mean age of the sample was 63.3 years (DS = 10.5), with a range of 33–83 years.

Yeast carriage

Thirty-nine subjects (75%) were carriers of yeast of *Candida*. Among them, 18 (69.2%) were compensated patients (HbA1c < 7%) and 21 (80.8%) were decompensated

Table 1. Diversity of yeast in the total sample of patients with carriage.

Specie	Total sample (%)	Subjects (n/%)	HbA1C ≤ 7%	HbA1C > 7%
C. albicans	66.1%	38 (97.4%)	18	20
C. glabrata	20.7%	11 (28.2%)	6	5
C. dubliniensis	8.23%	1 (2.56%)	0	1
C. tropicalis	1.73%	3 (7.69%)	1	2
C. guilliermondii	0.96%	2 (5.13%)	1	1
Rhodotorula spp.	0.02%	1 (2.56%)	0	1

HbA1c: glycosylated haemoglobin.

(HbA1c > 7%); however, this difference was not statistically significant.

Number of colony forming units

Eighteen (46.1%) subjects showed values lower than 400 CFU/ml and 21 (53.9%) showed values higher than 400 CFU/ml. The average CFU/ml was 2293 (SD = 3702), and ranged from 0 to 11,700 CFU/ml. Sixteen compensated subjects (61.5%) had values less than 400 CFU/ml, and 10 (38.5%) had values greater than 400 CFU/ml. In the decompensated group, eight (30.8%) subjects had values less than 400 CFU/ml, and 18 (69.2%) had values greater than 400 CFU/ml (*p* = .025).

Yeast species

Six yeast species were identified. The most frequent was *C. albicans* (66.1%), followed by *C. glabrata* (20.7%), *C. dubliniensis* (8.23%), *C. tropicalis* (1.73%), *C. guilliermondii* (0.96%) and *Rhodotorula spp* (0.02%). The remaining 2.21% of the samples could not be identified. Thirty-eight subjects (97.4%) had *C. albicans,* 11 (28.2%) had *C. glabrata,* three (7.69%) had *C. tropicalis*, two (5.13%) had *C. guilliermondii* and one (2.56%) had *C. dubliniensis.* One patient had an unidentified species of the genus *Rhodotorula spp.* (Table 1).

Diversity and coexistence of yeast species

Twenty-five (48.1%) subjects had only one *Candida* species, eight (15.4%) had two species, and six (11.5%) had three different species. Thirteen (25.0%) subjects did not carry yeast. In the compensated group, 12 (16.1%) subjects had one species of yeast, four (15.4%) had two species, and two (7.7%) had three different species. In the decompensated group, 13 (50.0%) subjects had one species of yeast, four (15.4%) had two species, and four (15.4%) had three species. The most common combination of coexisting yeasts was *C. albicans* and *C. glabrata* (42.9%), followed by *C. albicans, C. glabrata* and *C. tropicalis* (21.4%). In 14% of subjects, *C. albicans* was associated with other yeast species of the genus *Candida,* and in 7.14% of subjects, *C. albicans* was associated with *Rhodotorula spp* or unidentified species. Among the men, six (50.0%) had one species and one (8.3%) had three species, whereas among the women, 19 (47.5%) had one species, eight (20.0%) had two species and five (12.5%) had three species. No significant differences were observed (Table 2).

Salivary pH in the total sample

The average salivary pH was 7.54 (SD: 0.46). In the compensated group, the average was 7.61 (SD: 0.56), and in the decompensated group, it was 7.52 (SD: 0.54). The difference between carriers and non-carriers was statistically significant in the pH range 7.1–8 ($p = .004$).

Comparison of yeast count CFU/ml according salivary pH between compensated and decompensated patients

There was a higher yeast count in the decompensated patients at all saliva pH ranges. The differences between decompensated and compensated patients in yeast count at different pH ranges were statistically significant (Table 3).

pH and diversity of species present

Differences were observed in the distribution of yeasts among the four patient groups categorized by salivary pH

Table 2. Coexistence of species of yeast in the total sample of patients with carriage.

Species	Patients (n/%)
C. albicans + C. glabrata	6 (42.9%)
C. albicans + C. glabrata + C. tropicalis	3 (21.4%)
C. albicans + C. guilliermondii + C. glabrata	1 (7.14%)
C. albicans + Rhodotorula sp.	1 (7.14%)
C. albicans + Candida sp.	1 (7.14%)
C. albicans + C. glabrata + Candida sp.	1 (7.14%)
C. albicans + C. guilliermondii + Candida sp.	1 (7.14%)

Table 3. Comparison of yeast count in CFU/ml according to salivary pH between compensated and decompensated patients.

pH	HbA1c ≤ 7% (CFU/ml/ number of patients)	HbA1c > 7% (n) (CFU/ml/number of patients)	p value
6.5–7	177 (7)	978 (4)	$p < .01$
7.01–7.5	684 (5)	2471 (4)	$p < .01$
7.51–8	1528 (9)	704 (10)	$p < .01$
>8	7 (5)	1436 (8)	$p < .01$

HbA1c: glycosylated haemoglobin.

range. There was a greater diversity of species in those subjects with a salivary pH in the range of 7.1–8. In addition, C. glabrata occurred at a highest proportion in the lowest salivary pH category, with the proportion decreasing with respect to the other species proportions as pH increased. C. albicans was higher in the saliva samples of more alkaline pH (Figure 1).

Susceptibility of Candida albicans to nystatin according to pH

There was a negative correlation between the variants ($r = -0.2234$) that was statistically significant ($p = .01$), suggesting that at higher pH, C. albicans yeast is less susceptible to nystatin, whereas at lower pH, C. albicans is more susceptible to treatment. The differences in susceptibility among the different pH ranges were significant (Figure 2).

Discussion

The aims of this study were to establish the number and species of Candida according to metabolic control state and evaluate their resistance in relation to salivary pH. The results can help improve therapies for oral candidiasis in DM2, potentially increasing their effectiveness and accuracy. The HbA1c test was employed to identify the relationship between blood sugar control and Candida colonization. Recently, Zomorodian et al. [8] identified the relationships between number and diversity of Candida species and metabolic control in DM2. However, they collected samples by swabbing the oral mucosa and not by collecting saliva. Our study is the first to correlate metabolic control in DM2 with the number and diversity of Candida species and resistance by analysing salivary samples. Previous studies are inconsistent regarding the relationship between blood sugar level and Candida colonization. Although some studies have found a direct, significant correlation between glycosylated haemoglobin and oral Candida colonization [16], we found no relationship between HbA1c level and Candida carriage or
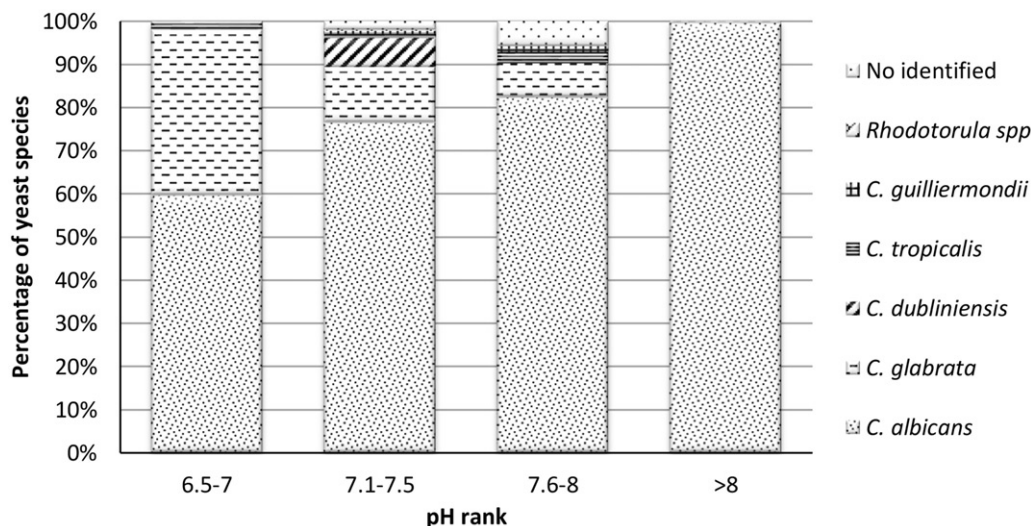

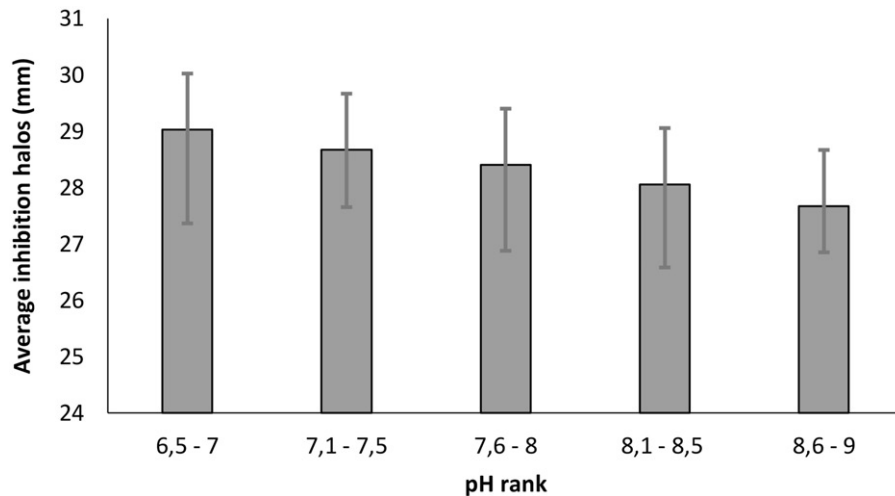Figure 1. Diversity of yeast species according to salivary pH in total salivary samples of patients with carriage.

Figure 2. Susceptibility of *C. albicans* to nystatin according to pH range. The *X*-axis presents the ranges of pH values. The *Y*-axis presents the average inhibition halo diameter (mm) and standard deviation produced by nystatin.

density in patients with DM, similar to other studies [17–19]. Several factors have been identified associated with the carrying of oral *Candida* in diabetic subjects, such as the years of disease duration, HbA1c level and local factors such as salivary pH [20]. Our results showed that 75.0% of subjects with DM2 carried *Candida* yeast. Evidence indicates that the prevalence of this yeast in subjects with DM2 ranges from 18 to 80% [21]. Discrepancies in prevalence estimates among studies might be attributed to differences in methodology, subject characteristics or sampling techniques. High levels of glucose in the saliva along with a decrease in salivary flow favour the growth of *Candida* yeast in diabetic patients [19]. Natural host cell receptors promote the adhesion of *Candida* to the epithelial surface. Salivary glucose form glycosylation products with proteins in tissues during episodes of hyperglycaemia [21], which increases the number of available epithelial receptors for *Candida* [22]. This phenomenon along with the decrease in salivary flow associated with diabetes favours *Candida* colonization [21]. Zomorodian et al. found that although the oral carriage of *Candida* species was significantly higher in diabetics than in controls, there was no direct association between *Candida* burden and glycosylated haemoglobin level in diabetics [8]. In our study, although a greater percentage of decompensated subjects than compensated subjects were carriers of *Candida*, this difference was not significant. Hammad et al. [16] described similar results. This may be because *Candida* is part of the normal oral microbiota independent of their systemic condition. A correlation has been reported between the presence of signs and symptoms of infection by *Candida* and yeast counts greater than 400 CFU/ml in saliva [23].

In our study, decompensated patients generally had counts greater than 400 CFU/ml. The carriage of *Candida* species can be higher in older patients than in younger ones, which might have been due to decreased salivary flow and the consumption of drugs associated with aging [24], however, in our study, it was not possible to establish this relationship. Since diabetes is a recognized risk factor associated with the development of candidiasis [25,26], the presence of high density of *Candida* yeasts in the oral cavity of diabetics should be considered a risk factor for infections as it could contribute to a deterioration of oral health over time. *Candida albicans* was the most often isolated species in our study, present in 97.4% of the yeast carriers. In addition, it represented 66.1% of CFUs. These values are similar to those reported in the literature, which describes this species as the most prevalent *Candida* species in the oral cavity of diabetic patients, with values between 67 and 87% [16,19,21]. These values might reflect the characteristics of *C. albicans*, i.e. its high capacity to adhere to the epithelial cells of the oral mucosa, generation of proteinases that yield high virulence, resistance to inflammatory host reaction and high secretion of phospholipases. The second most common species in our study was *C. glabrata*, representing 20.7% of the total sample of CFUs and occurring in 11 (28.2%) of the patients with yeast. A recent study indicated that *C. albicans* promotes invasion by *C. glabrata* [27]. In our study, the coexistence of these yeasts was observed in 16 subjects (30.8%). The synergistic relationships between *Candida* species can promote the colonization of more resistant strains, promoting infection and increasing disease severity [28].

DM2 subjects, regardless of their metabolic control, may have low salivary pH compared with that of non-diabetic patients. The greatest number and diversity of yeast species were observed in the pH range of 7.01–8, which indicates that most of the microorganisms present in the oral cavity require a pH near neutral to survive. We observed an inverse relationship between both the diversity and number of species of yeast in the saliva of patients with DM2 and salivary pH. An acid pH in the mouth favours the growth of yeasts over the growth of normal bacterial microbiota. Yeasts of the genus *Candida* tolerate very acidic pH values [6]. *Candida albicans* is capable of lowering the pH of the medium with high glucose [18]. Glucose in saliva also serves as a nutrient for *Candida* and suppresses the phagocytic ability of neutrophils, which further promotes *Candida* colonization [28]. Our results suggest the need to modify the therapeutic approach of fungal infections of the oral cavity of diabetic patients based on their salivary pH. Nevertheless, our sample could be small to establish conclusive results.

Resistance to antifungals is a serious issue. It is becoming increasingly difficult to treat candidiasis [29,30]. As *C. albicans* is the most common yeast species in diabetic patients, we studied resistance in only this species. Susceptibility testing of clinical yeast isolates is essential to optimize antifungal therapy and several methods have been standardized to obtain rapid and accurate results. Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility testing (EUCAST) have standardized broth microdilution susceptibility testing as gold-standard assays for yeast [31,32]. However, broth microdilution assays are often time-consuming especially for multiple strains analysis. For this reason, we evaluated susceptibility through classic disk diffusion method according to CLSI M44-A guide, which has the advantage of obtaining results faster and they are easier to interpretation. Recent studies have shown that results obtained from disk diffusion methods correlate with MICs obtained from broth microdilution assays and could be useful to estimate susceptibility of clinical strains [33]. On the other hand, M44-A guide has been standardized only for azoles so our results should be used with caution for nystatin.

For nystatin, greater inhibition was observed at lower pH values, which suggests that it might have greater effectiveness in patients with higher salivary acidification. Some studies indicate that DM2 patients having metabolic decompensation (HbA1c > 7%) exhibit greater salivary acidification [5], nevertheless, that tendency not observed in our study. With respect to fluconazole, the results were not significant. Several studies have evaluated the effects of different presentations of nystatin, such as mouthwash [34], nanocomposites [35] and as an ingredient of tissue conditioners [36]. It will be interesting to evaluate the effectiveness of these formats clinically in the future. Future studies are necessary to investigate drug interactions. It is reported that sulfonylureas, thiazolidinediones and meglitinides, which are used in DM, may have pharmacological interactions with fluconazole [37]. In addition, it is necessary to evaluate caries and periodontal disease in DM subjects because acid salivary pH is described in caries and alkaline pH is described in periodontitis [38]. The use of removable prostheses should also be considered because *C. albicans* has an exceptional ability to adhere to the surfaces of artificial materials [6]. Furthermore, it is necessary to assess susceptibility in the presence of species of *Candida* other than *C. albicans* and in cases of the coexistence of more than one species. The diversity of species detected in our study, many of which are resistant to azole antifungals, suggests the need to reevaluate treatment in diabetic subjects with candidiasis.

It must be considered in future, to make more consistent relationships between studied variables, increasing the number of sample individuals. The principal limitation of this study is the low number of patients especially in each salivary pH subgroups. In addition, it would also be useful, to assess the morphology of yeasts according to salivary changes in each individual, which might be associated with virulence and clinical manifestations of candidiasis. We consider that our results are a good initial background to begin to be reevaluating the antifungal therapies, specially in patients with diabetes mellitus, that can be much more effective if we consider in its indication, qualitative salivary characteristics as pH, easy to measure and as our results indicate, could determine the susceptibility of species of Candida to at least to nystatin, which is still the most used antifungal for the treatment of oral candidiasis in diabetic and non-diabetes patients.

Disclosure statement

Funding

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