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Short Communication

In vitro antifungal susceptibility of *Candida* species isolated from diabetic patients

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Abstract

Introduction: This study aims to evaluate the antifungal susceptibility of different species of *Candida* isolated from diabetic patients against eight antifungal agents. **Methods**: Susceptibility testing of 111 clinical isolates of *Candida* species was performed against 8 antifungals using the M27-A3 protocol of the Clinical and Laboratory Standards Institute (CLSI). **Results**: Voriconazole, lanoconazole, and caspofungin showed the highest *in vitro* activity against all the isolates of *C. albicans*. Resistance against the tested antifungals was only observed in the *C. albicans* isolates. **Conclusions**: Our finding revealed that resistance against amphotericin B, itraconazole, ketoconazole, posaconazole, and fluconazole can be observed in *C. albicans*.

Keywords: Diabetes. *Candida*. Antifungal susceptibility test.

Diabetes mellitus (DM) is the most prevalent type of diabetes and leads to harmful effects on multiple organs. Diabetes is prevalent among all age groups and has been predicted to show an increase from 171 million cases in 2000 to 366 million cases in 2030[1]. DM patients are known to be susceptible to infections. *Candida* species, especially *Candida albicans*, are a part of the normal flora of the oral cavity, intestinal tract, vagina, and skin in healthy individuals[2]. DM can be the underlying disorder for environmental changes of the oral cavity and provide favorable conditions for candidal colonization and cause an infection. This can result in a wide variety of clinical manifestations from superficial to systemic infections caused by different species of *Candida*[2]. Oral colonization and a high density of *Candida* species is more common among diabetic patients than non-diabetics[3]. Although *C. albicans* is considered the most common cause of candidal infections, the prevalence of non-*albicans* species has recently increased[4]. On the other hand, reports on the trends in the rates of resistance to azoles by *Candida* species isolated from patients with diabetes are increasing. This has been seen particularly in *C. albicans* that are typically azole-susceptible[5]. This epidemiologic shift is greatly impacted by pre-exposure to broad-spectrum azoles in patients who receive these agents either as antifungal therapy or prophylactic agents[4]. Accordingly, in this study, we aimed to evaluate the antifungal susceptibility of different isolated species of *Candida* from diabetic patients[6] against eight antifungal agents.

From February 2014 to June 2014, 300 patients with DM from Mazandaran, a Northern Province of Iran, were included in the study. The patients with any pre-existing fungal infections were excluded. The patients gave informed consent to participate in the research, and the study design was approved by the ethics committee of the Mazandaran University of Medical Sciences. All the isolates were cultured on Sabouraud's Dextrose Agar (Difco Laboratories Detroit, MI, USA) supplemented with chloramphenicol (0.5mg/mL) (SC). The plates were incubated at 27 - 30^{0}C for up to 7 days. The grown yeast-like colonies were identified to the species level by restriction fragment length polymorphism (RFLP) analysis, as described previously[7]. There was a modification in the procedure after the addition of the first restriction enzyme, *MspI* (Roche Molecular, Mannheim, Germany). To supplement the digestion of the polymerase chain reaction (PCR) products, a second restriction enzyme, *Bln1* (Fermentas, Germany), was added, after which the same procedure was followed.

Genomic deoxyribonucleic acid (DNA) was extracted as per the phenol-chloroform protocol after the disruption of the yeast cells by glass beads, as described previously[6].

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Antifungal susceptibility testing was performed using broth microdilution based on the M27-A3 protocol of the Clinical and Laboratory Standards Institute (CLSI)[7]. *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) were used as the quality control species in all the experiments. All the isolates of the *Candida* species were examined against 8 antifungal agents including itraconazole (ITR), ketoconazole (KET), voriconazole (VOR), lanoconazole (LAN), fluconazole (FLU), amphotericin B (AMB) (Sigma-Aldrich, St. Louis, MO, USA), posaconazole (POS) (Schering-Plough B.V., Boxmeer, the Netherlands), and caspofungin (CAS) (Pfizer, Capelle aan den Ijssel, the Netherlands). AMB, ITR, VOR, POS, KET, and LAN were dissolved in dimethyl sulphoxide (DMSO) while FLU and CAS were dissolved in deionized water. Serial twofold dilutions of the drugs were carried out to obtain a final concentration between 64 to 0.13µg/mL for FLU and between 16 to 0.03µg/mL for the rest of the tested drugs. The antifungal agents were diluted in standard Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) buffered to pH 7.0 with 0.165mol L^{-1} morpholine propane sulfonic acid buffer with L-glutamine without bicarbonate (MOPS, Sigma-Aldrich, St. Louis, MO, USA). According to the CLSI protocol[7], the minimum inhibitory concentration (MIC) of each antifungal drug was evaluated after 24h at 35°C.

The MIC for susceptible (S), susceptible-dose dependent (SDD), and resistance (R) was defined according to the CLSI protocol[7] and the M27-S3 supplement of the CLSI[8]. The data was analyzed using Statistical Package for the Social Sciences (SPSS) software (version 19).

The patients' data was presented in our previous published study[9]. In brief, out of 300 patients, 224 (74.7%) were female. The mean age of the patients was 56.83 (range: 30 - 90) years. The 51 - 60 year age group had the most frequency (35.3%). According to the glycosylated hemoglobin (HbA1c) results, 52 (17.3%) DM patients were classified as suffering from controlled diabetes and 248 (82.7%) had uncontrolled diabetes. Of these two groups, *Candida* species were identified in 25% and 39.5% of patients with controlled and uncontrolled diabetes *(P*=0.143), respectively. Out of 300 patients, 111 (37%) cases were positive for *Candida* species growth. The *Candida* species were isolated from the oral mucosa (104), axilla (2), vagina (2), and the skin surfaces of chest area (3) of patients with diabetes. According to the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach, *C. albicans* (93.7%) was the most commonly isolated species, followed by *Candida parapsilosis* (2.7%), *Candida glabrata* (1.8%), and *Candida tropicalis* (1.8%). The geometric mean (GM) MICs, MIC_{50}, and MIC_{90} of ITR, KET, POS, VOR, LAN, FLU, CAS, and AMB against *Candida* isolates are summarized in **Table 1**. According to the number of each identified isolate from the patients, we considered only the evaluation of MICs obtained for *C. albicans* isolates. As shown in **Table 1**, VOR, LAN, and CAS showed the highest MICs against all the isolates of *C. albicans* with MICs ranging from 0.016 - 2µg/mL. Resistance against the tested antifungals was observed in the *C. albicans* isolates. The most resistant isolates of *C. albicans* were observed against AMB (6.7%). Resistant isolates were not observed among the non-*albicans* species of *Candida*.

Due to the limited number of *C. parapsilosis*, *C. glabrata,* and *C. tropicalis* isolates, the calculation of relevant MIC_{50}, MIC_{90}, and GM was not possible.

Accordingly, due to the lack of data on the clinical breakpoint of LAN, the determination of the S, SDD, and R isolates of *Candida* species against LAN could not be done.

In the present study, we evaluated 111 isolated species of *Candida* against eight antifungals. *C. albicans* was the only species which showed resistance against the tested antifungals as follows: FLU (1.0%), KET (2.9%), POS (2.9%), ITR (4.8 %), and AMB (6.7%). All the isolates of *C. albicans* were susceptible to VOR and CAS, however, the SDD was observed in 1.9% of *C. albicans* to VOR. Kowalewska et al[10] reported that susceptible strains to AMB and ITR were reported in 100% and 28% of *C. albicans* isolated from the fecal samples of children with type 1 diabetes mellitus, respectively. In a study carried out by de Aquino Lemos et al[11], all isolates of *C. albicans* (MIC ≤ 1µg/ml) showed a high susceptibility to AMB and CAS while only two isolates (6.4%) were resistant to FLU. Pfaller et al[12] also reported a high activity of CAS against the clinical isolates of *C. albicans*. However, there are also a few reports on the resistance of *Candida* species against amphotericin B[13]. Our findings corroborate these previously reported results regarding the efficacy of CAS against *Candida* species. Our results have also confirmed that CAS is more active than FLU against the clinical isolates of *C. albicans* (**Table 1**).

A remarkable point in our finding was the low MICs of LAN against all the isolates of *Candida* species. The MICs range and MIC_{90} of LAN against *C. albicans* were 0.016 – 2 and 1µg/mL, respectively. However, due to the lack of data on the clinical breakpoint of LAN, the determination of the S, SDD, and R isolates of the *Candida* species against LAN was not possible. LAN is known as a topical antifungal agent with activity against superficial mycoses especially dermatomycosis and cutaneous candidiasis[14]. In this study, the GM MIC of LAN against clinical isolates of *C. albicans* was 0.14µg/mL. Our findings showed a slight difference in the *in vitro* inhibition potency of LAN in comparison with that reported by Tatsumi et al.[15] The latter reported the GM MIC range of LAN against clinical isolates of *C. albicans* and several non-*albicans* species of *Candida* as 0.0625 - 1.59µg/mL.

Our findings have revealed that *C. albicans* isolated from diabetic patients exhibited resistance to some antifungals including AMB, ITR, KET, POS, and FLU, the main antifungal agents against superficial and systemic candidal infections. Therefore, we strongly recommend performing the antifungal susceptibility test for all the isolated species of *Candida* to optimize the treatment of candidal infections.

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Conflict of interest

The authors declare that there is no conflict of interest.

TABLE 1: Minimum inhibitory concentrations for of antifungal agents for *Candida* species determined by the CLSI broth microdilution methods.

MIC interpretation* (%)			MIC (µg/mL)				Antifungal agent	Species
R	SDD	S	GM	90%	50%	Range		
4.8	11.5	83.7	0.095	0.25	0.063	0.016 – 4	Itraconazole	*C. albicans* (n=104)
0.0	1.9	98.1	0.060	0.125	0.032	0.016 – 2	Voriconazole	
2.9	0.0	97.1	0.070	0.125	0.063	0.016 – 4	Posaconazole	
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6.7	12.5	80.8	0.126	0.5	0.125	0.032 – 4	Amphotericin B	
0.0	0.0	100.0	0.132	0.5	0.125	0.016 – 2	Caspofungin	
1.0	7.7	91.3	1.280	13.6	1	0.016 – 64	Fluconazole	
2.9	0.0	97.1	0.085	0.25	0.063	0.032 – 8	Ketoconazole	
0.0	0.0	100.0	-	-	-	0.125 – 0.125	Itraconazole	*C. glabrata* (n=2)
0.0	0.0	100.0	-	-	-	0.063 – 0.063	Voriconazole	
0.0	0.0	100.0	-	-	-	0.125 – 0.125	Posaconazole	
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0.0	0.0	100.0	-	-	-	0.125 – 0.25	Amphotericin B	
0.0	0.0	100.0	-	-	-	0.063 – 0.5	Caspofungin	
0.0	0.0	100.0	-	-	-	2 – 4	Fluconazole	
0.0	0.0	100.0	-	-	-	0.063 – 0.063	Ketoconazole	
0.0	0.0	100.0	-	-	-	0.063 – 0.125	Itraconazole	*C. parapsilosis* (n=3)
0.0	0.0	100.0	-	-	-	0.032 – 0.063	Voriconazole	
0.0	0.0	100.0	-	-	-	0.063 – 0.125	Posaconazole	
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0.0	33.3	66.7	-	-	-	0.032 – 0.5	Amphotericin B	
0.0	0.0	100.0	-	-	-	0.032 – 0.5	Caspofungin	
0.0	0.0	100.0	-	-	-	0.25 – 4	Fluconazole	
0.0	0.0	100.0	-	-	-	0.032 – 0.25	Ketoconazole	
0.0	50.0	50.0	-	-	-	0.063 - 0.32	Itraconazole	*C. tropicalis* (n=2)
0.0	0.0	100.0	-	-	-	0.032 – 0.032	Voriconazole	
0.0	0.0	100.0	-	-	-	0.032 – 0.032	Posaconazole	
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0.0	0.0	100.0	-	-	-	0.125 – 0.25	Amphotericin B	
0.0	0.0	100.0	-	-	-	0.25 – 0.25	Caspofungin	
0.0	0.0	100.0	-	-	-	1 – 2	Fluconazole	
0.0	0.0	100.0	-	-	-	0.032 – 0.125	Ketoconazole	

CLSI: Clinical and Laboratory Standards Institute; **MIC:** minimum inhibitory concentration; **GM:** geometric mean; **S**: susceptible; **SDD**: susceptible-dose dependent; **R:** resistance; ***C.:*** *Candida*. *The MIC for susceptible, susceptible-dose dependent, and resistance was defined according to the CLSI protocol[8] and the M27-S3 supplement of the CLSI[9].

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