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Antifungal Susceptibility of *Candida* Species Isolated from Cancer Patients with Oral Lesions Undergoing Chemotherapy

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Abstract

Background: Oral candidiasis is the most common opportunistic infection of the oral cavity in patients undergoing chemotherapy. Identification of *Candida* species and the corresponding susceptibility to antifungal agents can be helpful in the management of cancer patients.
Objectives: The purpose of this study was to determine the susceptibility patterns of *Candida* species against 3 antifungal agents and define clinical practice guidelines for the prevention and treatment of oral candidiasis in cancer patients.
Methods: A total of 12 positive samples of oral lesions caused by *Candida* species were isolated from patients undergoing chemotherapy through direct examination and culture on CHROMagar *Candida* medium. Stock cultures were grown on sabouraud dextrose agar and DNA extracts. Then, polymerase chain reaction (PCR) was performed and the products were sequenced. The microdilution method was applied at different concentrations of fluconazole, amphotericin B, and nystatin. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of each species were compared.
Results: The species distribution of *Candida* isolates was as follows: *C. albicans*, 6 (50%); *C. krusei*, 3 (25%); and *C. tropicalis*, 3 (25%). The male-to-female ratio was 8:4, and the mean age of cancer patients was 51.25 years (range, 25-81 years). Overall, 100% and 83% of *C. albicans* were resistant to nystatin and fluconazole, respectively. All *Candida* species showed the lowest and highest resistance to amphotericin B (8.3%) and nystatin (66.7%), respectively.
Conclusions: DNA sequencing showed that *C. albicans* is the most commonly identified species in the oral cavity of cancer patients. Amphotericin B, compared to fluconazole and nystatin, is a more suitable antifungal drug for oral candidiasis. Oral hygiene involves dental cleaning, and management of poor denture hygiene and xerostomia can be helpful in eliminating *Candida* species in patients undergoing chemotherapy.

Keywords: *Candida*, Oral Candidiasis, Chemotherapy, Cancer, Antifungal Susceptibility

1. Background

Oral candidiasis, as an opportunistic infection of the oral cavity, is recognized as the most common human fungal infection. More than 80% of clinical infections associated with oral candidiasis are caused by *Candida* species, including *C. albicans*, *C. krusei*, *C. glabrata*, and *C. tropicalis* (1). The incidence of infection with *C. albicans*, as the most important fungal pathogen isolated from the oral cavity, has been reported to be 30% - 65% in neonates, children, and healthy adults. Also, the incidence rates have been reported at 65% - 88% and 90% - 95%, respectively in long-term care facilities (with antibiotic prescription) and patients with HIV/AIDS and acute leukemia undergoing chemotherapy (2-4). In the general population, 20-75% of the reported cases have shown no symptoms of infection (1).

Candida infections can also be a marker of malignancy and disease among immunocompromised individuals. Reactive oxygen species seem to cause oral mucositis, given exposure to radiation or chemotherapy (5). Oral microbial flora is stable in healthy people, but changes in cancer patients undergoing chemotherapy. In fact, it may expand through the bloodstream or upper gastrointestinal tract, leading to serious systemic diseases and increased morbidity and mortality rates (up to 79%); therefore, prompt diag-

nosis and adequate therapy are necessary (2, 6).

Despite the nephrotoxicity (a dose-limiting factor) associated with its use and different infusion-related side effects, amphotericin B is the main drug for the treatment of serious fungal infections. Azoles are effective and safe agents for the treatment of oral lesions caused by *Candida* species and have gradually replaced amphotericin B (7). In case of resistance to azoles, nystatin can be used as a suitable antifungal agent in cancer patients to eradicate *Candida* colonization in the mouth (8). However, in Iran, in vitro antifungal testing of clinical isolates is not routinely performed to guide the selection of antifungal therapy.

2. Objectives

The purpose of this study was to determine the susceptibility patterns of *Candida* species against 3 antifungal agents and define clinical practice guidelines for the prevention and treatment of oral candidiasis in cancer patients.

3. Methods

3.1. Sampling and Culture Conditions

A total of 12 positive samples of oral lesions caused by *Candida* species were isolated from patients undergoing chemotherapy at Seyed Al-Shohada hospital, Isfahan, Iran. All patients completed the consent forms, and the study was approved by the research ethics committee of Isfahan University of Medical Sciences. Also, a questionnaire was developed to record the medical history of patients, type of cancer, and demographic data.

All the specimens from the oral lesions were collected using 2 sterile swabs and were transferred to tubes containing 0.5 mL of saline solution. The swabs were used for direct examination and culture studies with incubation at 35°C for 48 hours on CHROMagar *Candida* medium (CHROMagar Company, France). Stock cultures were grown on sabouraud dextrose agar (SDA; Biolife, Italy) and were incubated at 35°C for 24 hours.

3.2. DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

DNA extraction was performed using the bulling method. For molecular identification, PCR was performed using the following primers as described by Shokohi et al. (9): ITS1, 5' TCCGTAGGTGAACCTTGC GG3' and ITS4, 5' TCCTCCGCTTATTGATATGC3'. The amplified products were visualized through 1.5% agarose gel electrophoresis in tris/borate/EDTA (TBE) buffer.

3.3. DNA Sequencing

To confirm the identified *Candida* species, 12 PCR products were sequenced and compared with the available sequences in GenBank, using the BLAST algorithm and the national center for biotechnology information database (www.ncbi.nlm.nih.gov).

3.4. Antifungal Susceptibility Testing

The antifungal drugs, including amphotericin B, fluconazole, and nystatin, were supplied as standard powders (Sigma-Aldrich, USA). Stock solutions of the drugs were prepared in dimethyl sulfoxide (DMSO) and methanol. The susceptibility patterns of the isolates were determined using broth microdilution assay, according to the clinical and laboratory standards institute (CLSI) M27-A3 guidelines (10); the tests were performed in triplicate. The final concentrations ranged from 0.0313 to 16 μg/mL for amphotericin B and nystatin and from 0.125 to 64 μg/mL for fluconazole.

The aliquots (100 μL) of each antifungal drug at a concentration twice as high as the target final concentration were dispensed in 96 wells of sterile microdilution plates. Yeast inoculum (at least 5 colonies) was suspended in 5 mL of sterile saline (0.85%). The turbidity of each suspension was adjusted spectrophotometrically at 530 nm to match 0.5 McFarland standard (corresponding to 1 - 5 $\times 10^6$ cells/mL). The inoculum was diluted in RPMI 1640 medium (supplemented with L-glutamine and glucose), resulting in 5.0 $\times 10^2$ - 2.5 $\times 10^3$ cells/mL.

A constant volume (100 μL) of the inoculum was added to each microdilution well, containing 100 μL of the serial dilution of antifungal agent to reach the final concentration. Also, 1 negative control with no fungal suspensions and 1 positive control with no drugs were used in each series. The plates were sealed and incubated at 35°C for 48 hours. Finally, the visual minimum inhibitory concentration (MIC) endpoints were determined. The MICs for amphotericin B, fluconazole, and nystatin were compared with the CLSI interpretative guidelines on antifungal susceptibility testing.

Fungal growth was considered susceptible at fluconazole doses ≤ 8 μg/mL, susceptible-dose-dependent at doses of 16 - 32 μg/mL, and susceptible at doses $\geq$ 64 μg/mL. Moreover, for amphotericin B, fungal growth was considered susceptible at doses < 1 μg/mL and resistant at doses $\geq$ 2 μg/mL. Also, for nystatin, fungal growth was considered susceptible at doses ≤ 16 μg/mL and resistant at doses > 16 μg/mL. Finally, the minimum fungicidal concentration (MFC) was measured.

4. Results

PCR amplification was successfully performed on 12 oral lesions of cancer patients undergoing chemotherapy. Also, DNA sequencing was successfully performed, and the species distribution of *Candida* isolates was identified: *C. albicans*, 6 (50%); *C. krusei*, 3 (25%); and *C. tropicalis*, 3 (25%). The male-to-female ratio was 8:4, and the mean age of cancer patients was 51.25 years (range, 25 - 81 years).

Comparison of in vitro susceptibilities (MIC and MFC) of *Candida* isolates to antifungal agents is presented in Table 1. The results showed that 100% and 83% of *C. albicans* were resistant to nystatin and fluconazole, respectively. All species showed the lowest and highest resistance to amphotericin B (8.3%) and nystatin (66.7%), respectively.

5. Discussion

Monitoring of antifungal resistance patterns of *Candida* species in cancer patients can present important information about major differences among different populations in terms of susceptibility patterns and fungal species distribution. Therefore, identification of *Candida* species and their susceptibility to antifungal agents can be helpful in the management of cancer patients (11).

There are 3 pathways for *Candida* resistance to drugs in a cancer patient: mutation in susceptible *Candida* species, infection with an inherently resistant species, and infection with more than 1 *Candida* species and an inherently resistant species (12). Higher mortality rates have been described in infections caused by organisms resistant to antibiotics and antifungals (13).

In previous research, *C. albicans* was the predominant organism isolated from cancer patients with oral candidiasis, leading to increased mortality under certain circumstances (14). In the present study, *C. albicans* was identified using the molecular method and was found to have the highest prevalence among cancer patients; this finding was similar to the results reported in previous studies (4, 15, 16).

The frequency of *Candida* species in cancer patients is associated with the type of cancer, immune system, and antibiotic resistance rates (17). The increasing incidence of colonization with *Candida* species due to decreased susceptibility to first-generation azoles (such as fluconazole) suggests the use of newer antifungal drugs. However, fluconazole with good absorption in the gastrointestinal tract and favorable oral safety and nystatin, used as a topical agent with few side effects, have been extensively used for chemoprophylaxis and treatment of fungal infections in severely immunodeficient patients (4, 18).

The importance of non-*C. albicans* species is associated with increased resistance to azoles, including fluconazole, especially in advanced cancer patients (19, 20). In this study, 100% and 83% of *C. albicans* and 33.3% and 16.7% of non-*C. albicans* isolates were resistant to nystatin and fluconazole, respectively. In previous studies, low rates of resistance to both drugs have been reported. In fact, the high resistance rate can be due to the indiscriminate use of antibiotics and antifungal drugs in immunodeficient patients.

Polyene antifungals, including amphotericin B, play a well-defined role as antifungal agents in patients who are unresponsive to broad-spectrum antibiotic therapy. Amphotericin B is not absorbed by the gastrointestinal tract and is used in topical applications for oral candidiasis (1). In the present study, this drug showed substantial activity against isolates with in vitro resistance to fluconazole and nystatin. These results suggest the activity of amphotericin B against oral *Candida* isolates, particularly those with reduced susceptibility to nystatin and fluconazole; the findings are similar to those reported by Shokohi and colleagues (9).

In conclusion, decreased effectiveness of antifungal drugs is a serious issue, especially in cancer patients. Amphotericin B, compared to fluconazole and nystatin, is a more suitable antifungal drug for oral candidiasis. Oral hygiene involves dental cleaning, and management of poor denture hygiene and xerostomia can be helpful in eliminating *Candida* infections in patients undergoing chemotherapy.

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Footnotes

Authors' Contribution: Experimental design of the study: Zahra Jabalameli, Ali-Mohammad Sabzghabaee, Hossein Safavizadeh, and Parvin Dehghan; implementation of the study, mycological examination, and sample collection, Zahra Jabalameli, Mehrnoosh Maherolnaghsh, and Mohammad-Ali Mohaghegh; review and contribution to the writing of the manuscript, Mohammad-Ali Mohaghegh, Zahra Jabalameli, and Parvin Dehghan.

Conflicts of Interest: There are no conflicts of interest.

Table 1. Antifungal Susceptibilities of Clinical *Candida* Isolates from Cancer Patients Undergoing Chemotherapy

Candida species	Accessions Number	Cancer Type	Age	Sex	Antifungal Agents								
					F●			A●			N●		
					MIC	MFC	S†	MIC	MFC	S†	MIC	MFC	S†
C. albicans	KY101883.1	Leukemia	54	Male	> 64	-	R*	0.001	8	S	> 16	-	R
	KY101874.1	Leukemia	27	Female	> 64	-	R	0.001	8	S	> 16	-	R
	KP675681.1	Bladder	51	Male	> 64	-	R	64	-	R	> 16	-	R
	KP675109.1	Bladder	46	Male	> 64	-	R	1	32	S	> 16	-	R
	KP675087.1	Lung	34	Female	> 64	-	R	1	16	S	> 16	-	R
	KP675450.1	Lymphoma	25	Male	1	8	S*	0.125	1	S	> 16	-	R
C. glabrata	KP674954.1	Breast	78	Female	> 64	-	R	0.062	2	S	8	-	S
	GU199447.1	Gastrointestin tract	57	Male	16	-	S	1	8	S	16	-	S
	KP878250.1	Lymphoma	53	Male	64	-	S	0.015	0.25	S	2	16	S
C. krusei	EU315756.1	Liver	59	Male	16	-	S	1	8	S	> 16	-	R
	AB467300.1	Lymphoma	81	Female	0.125	-	S	0.25	32	S	0.064	1	S
	EU315751.1	Gastrointestin tract	50	Male	2	2	S	0.001	0.5	S	> 16	-	R

Abbreviations: F●, fluconazole; A●, amphotericin B; N●, nystatin; S†, sensitivity; R*, resistant; S*, sensitive.

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