Original article

In vitro antifungal susceptibility of *Candida* species isolated from oropharyngeal lesions of patients with cancer to some antifungal agents

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Abstract

Introduction and objective: Oropharyngeal candidiasis is a relatively common mycotic infection in cancer patients. *In vitro* susceptibility of oropharyngeal *Candida* isolates can be useful in selecting the appropriate treatment for the best therapeutic outcome. The aim of this study was to evaluate the *in vitro* antifungal activity of *Candida* species against antifungal agents.

Material and methods: *In vitro* activities of four antifungals were detected in 69 *Candida* isolates recovered from cancer patients in four university hospitals using the microdilution method described in the CLSI M27-A3 guideline.

Result: Only 12(17.4%) of *Candida* isolate were resistant to antifungal agents. Three isolates (4.4% included *C. albicans*, *C. glabrata, C. tropicalis,* and *C. pelliculosa)* were resistant to amphotericin B, 5(7.2% included two *C. albicans,* two *C. glabrata,* and one *C. kefyr*) were itraconazole resistant. Two (2.9% include one *C. albicans* and one *C. glabrata*) were fluconazole resistant. Caspofungin resistance was detected in two *C. infanticola* strains which were reported as a clinical isolate for the first time. All *Candida* isolates (n=69) taken together gave minimum inhibitory concentration (MIC_{90}) value for amphotericin B, fluconazole, itraconazole and caspofungin of 1, 0.25, 32 and 0.25µg/ml, respectively. In total, 18.7% of *C. glabrata* and 7.8% of *C. albicans* isolates were fully resistant to both itraconazole and fluconazole.

Conclusion: Caspofungin had activity against oropharyngeal non- *albicans Candida* species isolates, particularly against those with reduced susceptibility to amphotericin B, fluconazole and itraconazole.

Keywords: Antifungals; *In vitro* susceptibility; *Candida* species; Oropharyngeal candidiasis

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Introduction

Candida spp. and *Candida albicans* are part of normal micro flora of oral cavity in 40% to 60% of healthy individuals. However, they are also opportunistic pathogen in immunocompromised patients. They can become aggressive, induce oropharyngeal candidiasis (OPC), invade the digestive tract and even lead to systemic infections [1]. OPC is a relatively common mycotic infection in cancer patients undergoing radiotherapy and/or chemotherapy [2-5].

Amphotericin B is the main therapy for serious fungal infections for more than 40 years. Infusion-related side effects and dose-limiting nephrotoxicity associated with its use prompted continuous search for equally effective but less toxic alternatives. Azoles are safe and effective agents for the treatment of oropharyngeal candidiasis and have gradually replaced amphotericin B. However, resistance to azoles is now becoming common. Several reports suggest that susceptibility rates of *Candida* spp. to triazole antifungal amongst cancer patients have remained high with fluconazole resistance restricted to *C. krusei* [6,7] and *C. glabrata* [6-8]. Other investigators have notified that *C. albicans* isolates from HIV positive and cancer patients are resistant to fluconazole and itraconazole [9,10].

Newer antifungals, such as caspofungin, have a broader spectrum of activity that includes fluconazole resistant *Candida* spp. and they are good substitutes for amphotericin B that cannot be tolerated by some patients. The use of this new option should therefore be considered for the treatment of refractory oropharyngeal candidiasis [11]. Although *C. albicans* is the most frequently implicated pathogen, other *Candida* species also may cause infection. The emergence of antifungal resistance within these causative yeasts, especially in patients with recurrent oropharyngeal infection or with long-term use of antifungal therapies, requires an urgent need for laboratory support in the treatment of fungal infections including [1] the rapid identification of fungal pathogens to the species level, and where appropriate [2] *in vitro* susceptibility testing of clinical isolates to guide the selection of antifungal therapy [12]. In this study, an attempt has been made to determine susceptibility pattern of four antifungal agents against the *Candida* species isolated from patients with cancer.

Materials and methods

Fungal strains and culture conditions

Sixty nine strains of *Candida* species were isolated from orophareangeal lesions of patients with cancer admitted in four university hospitals of Mazandaran province. All patients gave written informed consent and the study was approved by the ethics committee of research deputy of Mazandaran university of Medical Sciences. These strains were previously identified by phenotypic methods such as germ tube formation in horse serum, chlamydospore formation in Corn meal agar-Tween 80 (Merck, Germany), colored colony morphology on CHROMagar Candida medium (CHROMagar Company, France), API 20C Aux (bioMerieux-France) and genotypic methods such as sequencing D1/D2 region of LSU r DNA gene [13] and RFLP-PCR region of ITS1-5.8S-ITS2 rDNA region [14]. Stock cultures and controls were grown on Sabouraud dextrose agar (SDA, (Merck, Germany)), and were incubated at 35°C for 24h.

Antifungal agents

The following agents were supplied as standard powders: Amphotricin B (Sigma-Aldrich, USA), fluconazole (Sigma-Aldrich, USA), itraconazole (Sigma-Aldrich, USA), and caspofungin (Merck, USA) and were dissolved in dissolved in

dimethyl sulfoxide (DMSO) (Sigma, USA) to make stock solutions.

Antifungal susceptibility testing

Antifungal susceptibility testing of the isolates was performed by micro broth dilution technique in accordance with Clinical and Laboratory Standard institute (CLSI) guidelines M27-A3 and M27-S3 [15,16]. All the testing was done in duplicates. The range of concentrations tested was 0.125-64μg/ml for fluconazole, 0.015-8μg/ml for caspofungin and 0.0313-16μg/ml for amphotericin B and itraconazole.

Aliquots of 100μl of each antifungal agent at a concentration two times the targeted final concentration were dispensed in the wells of the sterile, disposable and flat bottom 96 well microdilution plates. Yeast's inoculum was prepared onto Sabouraud dextrose in sterile saline (0.85%) after 24h of incubation, obtaining an initial concentration of 1 to 5×10^6 cell/ml (adjusted spectrophotometrically at 625nm to match the turbidity of a 0.5 Mc Farland standard). This inoculum was diluted in RPMI 1640 medium with L-glutamine, without sodium bicarbonate (Sigma, USA) buffered with morpholine- propanesulfonic acid (Sigma-Aldrich, Germany) in order to obtain a final concentration of 0.5×10^3 to 2.5×10^3 cell/ml.

A constant volume (100μl) of the inoculum was added to each microdilution well containing 100μl of the serial dilution of antifungal agents to reach final concentrations. The microplates were incubated at 35°C for 48h. Visible fungal growth can be inhibited 100% by the MIC_{100} of amphotericin B, whereas it can be inhibited 50% by $MICs_{50}$ of azoles and caspofungin in relation to the drug-free growth control. In each test *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were used for quality control.

The minimum inhibitory concentration (MIC) values for fluconazole, itraconazole, and amphotericin B were compared to the CLSI interpretative guideline on antifungal susceptibility testing or based on CLSI standard protocol [15,16]. The fungal growth is considered as resistant when ≥64μg/ml of fluconazole is used, it is considered as susceptible dose dependent when16-32μg/ml of fluconazole is used, and is considered as susceptible when ≤8μg/ml used. The fungal growth is considered as resistant when ≥1μg/ml of itraconazole is used, it is considered as susceptible dose dependent when 0.25-0.5μg/ml of itraconazole is used, and is considered as susceptible when ≤0.125μg/ml used. The fungal growth is considered as resistant when ≥2μg/ml of amphotericin B is used, and is considered as susceptible when <1μg/ml used. The fungal growth is considered as resistant when ≥2μg/ml of caspofungin is used, and is considered as susceptible when ≤2μg/ml used. The MICs endpoints were determined with reading mirror.

Results

A total of 69 oropharyngeal *Candida* isolates from patient with cancer which were previously identified were included in this study. The species distribution of *Candida* isolates were *C. albicans* (38, 55%), *C. glabrata* (16, 23.2%), *C. tropicalis* (6, 8.7%), *C. parapsilosis* (1, 1.5%), *C. krusei* (2, 2.9%) and *C. infanticola* (2, 2.9%), *C. kefyr* (1, 1.5%), *C. pelliculosa* (1, 1.5%), *C. orthopsilosis* (1, 1.5%), *C. terebra* (1, 1.5%). These two *C. infanticola* strains were isolated from the mouth of a two year-old boy and a 67 year-old man, both suffering from cancer, identified with molecular tools.

The comparative *in vitro* susceptibilities of the yeast isolate to the antifungal agents were shown in Table 1. MICs for *C.*

parapsilosis (ATCC 22019) and *C. krusei* (ATCC 6258) were within the expected ranges. Overall, 66(95.7%) of isolates were susceptible to amphotericin B (MIC≤1μg/ml). Three isolates (4.3% included *C. albicans, C. glabrata, C. pelliculosa)* were resistant to amphotericin B (MIC>1μg /ml). Thirty three (47.8%) of the isolates were susceptible to itraconazole (MIC≤0.125μg/ml), 31(44.9%) were susceptible dose dependent and 5(7.2%) were itraconazole resistant (MIC≥1μg /ml). Fifty four (78.2%) of the isolates were susceptible to fluconazole (MIC≤8μg/ml), 13(18.4%) were susceptible dose dependent and 2(2.9%) were fluconazole resistant (MIC≥64μg/ ml).

All *Candida* isolates (n=69) taken together gave MIC_{90} value for amphotericin B, fluconazole, itraconazole and caspofungin of 1, 32, 0. 25, and 0.25μg/ml, respectively. Only 12(17.4%) of *Candida* isolates (four of *C. albicans*, four of *C. glabrata*, one of *C. pelliculosa*, one of *C. kefyr* and two of *C. infanticola*) were resistant to antifungal agents. Only 2(2.9%) and 3(4.3%) of the *Candida* isolates were resistant to caspofungin and amphotericin B, respectively.

In total, three of 16 *C. glabrata* isolates (18.7%) and three of 38 *C. albicans* isolates (7.8%) were fully resistant to both itraconazole and fluconazole. However, none of the *C. tropicalis, C. parapsilosis, C .krusei, C. orthopsilosis* and *C. terebra* was resistant to these four antifungals. Overall, 31(45%) of isolates were non-*albicans Candida* species which eight of them (25.8%) were resistant and 19 of them (61.2%) were susceptible dose dependent to antifungals. For all together non-*albicans Candida* species (n=31) MIC_{90} values for amphotericin B, fluconazole, itraconazole and caspofungin of 1, 16, 0.5 and 0.125μg/ml, respectively (not shown in table 1).

We found the widest range and the highest MICs for fluconazole (range 0.063-64). The isolates of *C. albicans* and *C. glabrata* had high MICs for fluconazole. Caspofungin and itraconazole demonstrated better in vitro activity than amphotericin B with MIC_{90} 0.25μg/ml, compared to 1μg/ml for all *Candida* isolates. In our study, some rare species that belong to *C. pelliculosa*, *C. krusei*, *C. orthopsilosis*, *C. parapsilosis*, *C. kefyr*, *C. terebra*, and *C. Infanticola* were identified at the basis of the sequence analysis.

Discussion

A variety of antifungal agents are now available for the treatment of oropharyngeal candidiasis infections. Amphotericin B is used in the treatment of superficial and systemic infections of hospitalized individuals [17]. This study demonstrated that *C. albicans* was the predominant species of oropharyngeal candidiasis in patients with cancer. Although *C. albicans* remain the most common pathogen in oropharyngeal candidiasis, non- *albicans* species are increasingly associated with invasive candidiasis [18]. Although triazole agents appear to be highly effective initially, the increase of resistance to them has been reported [17,19].

Decreasing susceptibility to the first generation azoles (fluconazole and itraconazole) due to the increasing incidence of colonization and infection with non- *albicans Candida* species, concerns have risen for newer antifungal drug. In the present study, overall resistance of all *Candida* isolates to fluconazole was 2.9%, which is similar to that reported by other previous studies [20, 21]. The reason for the low fluconazole resistance could be explained by the fact that fluconazole was not prescribed to the most cancer patients as a standard care in Iran.

In the present study, 5.4% of *Candida albicans* were resistant to itraconazole which is accordant with those investigators which reported itraconazole resistance 4% [22] and 7% [23] in advanced cancer and immunocompromised patients, respectively. In the present study 45% of isolates were non-*albicans Candida* species which 19.4% of them were resistant to amphotericin B, itraconazole and fluconazole. Also in this study 18.7% *C. glabrata* isolates were fully resistant to both itraconazole and fluconazole. Almost similar results were observed by Gonzales *et al*. [24] who showed 31.3% of *C. glabrata* bloodstream isolates were resistant to fluconazole, 43.3% were resistant to itraconazole. Badiee *et al*. [25] also noted resistance to fluconazole and itraconazole in some *Candida* species isolates from mucosal sites of HIV-positive patients in Shiraz, Iran.

Caspofungin showed substantial activity against isolates demonstrated in vitro resistance to fluconazole, itraconazole, and amphotericin B. These results suggest that caspofungin activity against oropharyngeal *Candida* isolates, particularly against those with reduced susceptibility to fluconazole and itraconazole. The results are similar to that reported by Pfaller *et al.* [26] who compared caspofungin with fluconazole and itraconazole against clinical isolates of *Candida* spp., including fluconazole-resistant isolates. They also showed that caspofungin was as active as or more potent than either fluconazole or itraconazole against all *Candid*a spp. with the exception of *C. guilliermondii* and *C. famata*. In our study there were no such *Candida* species between our isolates, but we demonstrated that caspofungin had no activity against *C. infanticola*.

Candida infanticola is a rare species that recently was isolated [27] from the ear of an infant in Germany. *C. terebra* is a rare species which was isolated from patients with allergic diseases [28]. *C. orthopsilosis* isolates which are phenotypically similar to *C. parapsilosis* has recently been distinguished by molecular methods [29-31]. This rare strain may be responsible for infection and colonization in humans. In our study, in accordance with Tavanti *et al*. [29] *C. orthopsilosis* isolate was found used to be susceptible to all antifungals.

This report further highlights the presence of emerging pathogens that could not be identified reliably and support the requirement for careful mycological identification at the species level of *Candida* isolates recovered from cancer patients, together with regular in vitro susceptibility testing to detect resistance to the azoles. These local surveillance studies can help clinicians make treatment decision. The data presented in this paper indicate that caspofungin and itraconazole are more effective than either amphotericin B or fluconazole against all non- *albicans Candida* species isolated from the oropharynx of patients with cancer. Thus, in such unresponsive case, caspofungin can be an alternative regime for managing oropharyngeal candidiasis.

Conclusion

These results suggest that caspofungin has activity against oropharyngeal non-*albicans Candida* species isolates, particularly against those with reduced susceptibility to amphotericin B, fluconazole and itraconazole.

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Table 1: The comparative *In vitro* susceptibilities of the *Candida* isolate from cancer patients to the antifungal agents

Species (N/%)	Antifungal	MICμg/ml			R*	SDD**
		50%	90%	Range	N (%)	N (%)
C .albicans	Amphotricin B	0.5	1	0.125-2	1(2.6)	-
(38/55)	Itraconazole	0.25	0.5	0.03-2	2(5.4)	18(48.6)
	Fluconazole	4	16	0.5-64	1(2.7)	6(15.7)
	Caspofungin	0.063	0.125	0.015-0.25	-	-
C. glabrata	Amphotricin B	0.5	1	0.125-4	1(6.2)	-
(16/23.2)	Itraconazole	0.25	0.5	0.063-4	2(12.5)	10(62.5)
	Fluconazole	2	16	1-64	1(6.2)	4(25)
	Caspofungin	0.031	0.125	0.015-0.25	-	-
C. tropicalis	Amphotricin B	0.25	0.5	0.125-2	-	-
(6/8.7)	Itraconazole	0.125	0.25	0.063-0.5	-	1(16.6)
	Fluconazole	1	4	0.25-16	-	-
	Caspofungin	0.063	0.5	0.031- 0.5	-	-
C. parapsilosis	Amphotricin B	0.25	0.5	0.25-0.5	-	-
(1/ 1.5)	Itraconazole	0.125	0.25	0.125-0.25	-	-
	Fluconazole	4	8	4-8	-	-
	Caspofungin	0.25	0.25	0.25-0.5	-	-
C. krusei	Amphotricin B	0.25	0. 5	0.25-0.5	-	-
(2/2.9)	Itraconazole	0.063	0.125	0.063-0.125	-	-
	Fluconazole	1	2	1-2	-	-
	Caspofungin	0.125	0.25	0.125-0.25	-	-
C. infanticola	Amphotricin B	0.125	0.125	0.125	-	-
(2/2.9)	Itraconazole	0.5	0.5	0.5	-	2(100)
	Fluconazole	4	8	4-8	-	-
	Caspofungin	4	4	4	2(100)	
C. orthopsilosis	Amphotricin B			0.25	-	-
(1/1.5)	Itraconazole			0.125	-	-
	Fluconazole			16	-	1(1.5)
	Caspofungin			0.5	-	-
C. pelliculosa	Amphotricin B			2	1(1.5)	-
(1/1.5)	Itraconazole			0.125	-	-
	Fluconazole			32	-	1(1.5)
	Caspofungin			1	-	
C. kefyr	Amphotricin B			0.5	-	-
(1/1.5)	Itraconazole			2	1(1.5)	-
	Fluconazole			16		-
	Caspofungin			1		-
C. terebra	Amphotricin B			0.25	-	-
(1/1.5)	Itraconazole			0.125	-	-
	Fluconazole			16	-	-
	Caspofungin			0.5	-	-
All *Candida*	Amphotricin B	0.5	1	0.125-2	3(4.4)	-
(69/100)	Itraconazole	0.125	0.25	0.03-4	5(7.2)	31(44.9)
	Fluconazole	4	32	0.063-64	2(2.9)	13(18.4)
	Caspofungin	0.125	0.25	0.015-4	2(2.9)	-
C. parapsilosis	Amphotricin B	1	1	1		
(ATCC 22019)	Itraconazole	0.5	0.5	1		
	Fluconazole	2	4	0.5		
	Caspofungin	1	1	1		
C. krusei	Amphotricin B	1	2	1-2		
(ATCC 6258)	Itraconazole	0.5	1	0.5-1		
	Fluconazole	32	68	32-68		
	Caspofungin	0.5	0.5	0.5		

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