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Full Length Research Paper

Investigation of the susceptibility of *Candida* species isolated from denture wearers to different antifungal antibiotics

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The aim of this study was to determine the the prevalence of *in vitro* resistance amongst *Candida* species isolated from the oral cavity of denture wearers. The *in vitro* susceptibility of 156 *Candida* isolates to amphotericin B, fluconazole, 5- fluorocytosine, caspofungin and terbinafine was determined. The Clinical Laboratory Standards Institute' (CLSI; formally National Committee for Clinical Laboratory Standards) broth microdilution method was used and MIC_{50} and MIC_{90} determined. *Candida albicans,* the most frequently isolated strains, are sensitive to amphotericin (61%) and fluconazole (44%), frequently used agents in the treatment of *Candida*-associated denture stomatitis. A 100% susceptibility to 5- fluorocytosine was observed among the 109 isolates of *C. albicans*. Among non *C. albicans* strains only 1 *Candida kefyr* strain was determined as susceptible dependent upon dose for 5-fluorocytosine. Among *Candida glabrata*, the second most common isolate, a 100% susceptibility to caspofungin and 5-fluorocytosine were observed. Since the isolates are sensitive to Caspofungin and 5-fluorocytosine, rarely used in the treatment of oral fungal infections, it is suggested that these antifungal agents be used as alternative medicine in the treatment of oral infections especially caused by strains resistant to amphotericin B and fluconazole.

Key words: *Candida* species, antifungal, denture wearers.

INTRODUCT ON

Since *Candida* species, which are among the normal flora microorganisms of human body, are opportunist pathogens, they can cause different clinical manifestations of candidiasis (Scardina et al., 2007). Oral candidiasis is the most common mycotic infection in the oral cavities in humans. Long-term use of prosthesis is the most important risk factor for *Candida* species colonization of the mucosal surfaces, this may be sufficient for the development of oral candidiasis. Oral fungal infection that causes *Candida* associated prosthesis stomatitis is a common disease in 50 to 60% of denture wearers (Budtz-Jorgensen, 2000; Darwazeh et al., 2001; Pıres et al., 2002).

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There are several antifungal medicines which can be used topically or systemically in the treatment of oral candidiasis. (i) Polyenes (amphotericin B and nistatin) form complexes with ergosterol which open channels in the fungal membrane that cause leakage of critical intracellular constituents and subsequent cell death. (ii) Azoles (fluconazole, itraconazole, ketoconazole, etc) which inhibits cellular membrane formation by interfering with ergosterol synthesis. (iii) Another chemical employed against *Candida albicans* is 5-fluorocytosine whose entry to the cell is mediated by the cytosine permease. This compound is transformed to 5- fluorourasil by cytosine deaminase. Incorporation of 5-fluorourasil into RNA interrupts protein synthesis leading to cell death (Ghannoum and Rice, 1999; Farah et al., 2000; Casalınuovo et al., 2004). The purpose of this work was to determine the minimum inhibitory concentration (MIC)

of amphotericin B, fluconazole, 5- fluorocytosine, caspofungin, terbinafine for *Candida* species isolated from individuals wearing prosthesis.

MATER ALS AND METHODS

Yeast strains

156 isolates of *Candida* spp. were previously isolated from saliva samples, smear from palatal mucosa and dorsum of the tongue were taken from total of 110 individuals (being treated for prosthodontic treatment in Ege University, Dental Faculty) -30 individuals wearing total prosthesis, 30 removable partial prostheses, 30 fixed prostheses and 20 with natural teeth. *Candida* species isolated were identified by germ tube and clamydospores production, and commercially available API 20C AUX yeast identification system (BioMerieux, France) (Abaci et al., 2010). Prior to antifungal susceptibility testing, all *Candida* spp. were subcultured at least twice on Sabouraud dextrose agar (SDA) plates.

Quality control

Quality control was ensured by testing the CLSI recommended quality control strains *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258.

Antifungal agents

Standard antifungal powders of amphotericin B (Bristol-Myers Squibb, Middlesex, UK), fluconazole (Sigma- RBI F8929), 5-fluorocytosine (Fluka, 4685), caspofungin (Merck, Co., Whitehouse Station, Pa.), and terbinafine (Santa Farma Ilaç San. A.S., Turkey) were obtained from their respective manufacturers. Stock solutions were prepared in water (caspofungin, fluconazole and 5-fluorocytosine) and amphotericin B was dissolved with DMSO. Serial twofold dilutions were prepared exactly as outlined in CLSI ducument M27-A2. Final dilutions were made in RPMI medium (Sigma-Aldrich, R65504) buffered to pH 7.0 with 0.165 M MOPS boffer [3-(*N*-morpholino) propanesulfonic acid] (CLSI M27-A2, 2002). Terbinafine was first dissolved at 100 fold highest final concentration in DMSO containing 5% Tween 80, after which sequential twofold dilutions were made in DMSO followed by fivefold dilutions of each solution in RPMI medium (Moore et al., 2001).

Antifungal susceptibility studies

Broth microdilution (BMD) testing was performed in accordance with the quidelines in CLSI document M27-A2 by using the spectrophotometric method of inoculum preparation, an inoculum concentration of 1.5(±1.0) x10^3 cells/ml, and RPMI 1640 medium buffered with MOPS. A 0.1-ml yeast inoculum was added to each well of the microdilution trays.

The final concentrations of the antifungal agents were 0.0625 to 32 µg/ml for amphotericin B, 0.125 to 64 µg/ml for fluconazole, 0.125 to 64 µg/ml for 5- fluorocytosine**,** 0.007 to 8 µg/ml for caspofungin, and 0.025 to 128 µg/ml for terbinafine. The trays were incubated at 35°C, and MIC were read after 48 h. Drug-free and yeast free controls were included.

Following incubation, the BMD wells were examined with aid of a reading mirror and the growth in each well was compared the inhibition of growth control well. The MICs were read for fluconazole and caspofungin as the lowest concentration of antifungal that inhibited 50% growth of the organism detected visually. The MIC of amphotericin B was defined as the lowest drug concentration causing 100% inhibition of fungal growth, 5-fluorocytosine were defined as the lowest drug concentrations at least 80% inhibition (CLSI M27-A2, 2002). Terbinafine were defined as the lowest drug concentrations at least 80% inhibition (Ryder et al., 1998; Moore et al., 2001). Antifungal activity was expressed as the MIC of each isolate to the drug. The following resistance breakpoints were used according to CLSI or based on previous investigation.

(i) Amphotericin B: resistant, MICs 2 µg/ml; susceptible, 1 µg/ml (CLSI M27-A2, 2002).
(ii Fluconazole: resistant, MICs 64 µg/ml; susceptible dose dependent, 16 to 32 µg/ml; susceptible, 8 µg/ml(CLSI M27-A2).
(iii) 5-fluorocytosine: resistant, MICs 32 µg/ml; intermediate, 8 to 16 µg/ml; susceptible, 4 µg/ml (CLSI M27-A2).
(iv) Caspofungin: The CLSI-approved MIC breakpoint for caspofungin susceptibility is 2 g/ml. There is no intermediate or dose-dependent category (Brown and Traczewski, 2008).
(v) Terbinafine: Clinically relevant breakpoints are currently not available for terbinafine. However, Ryder et al. (1998) evaluated that a breakpoint of >8 µg/ml was taken to indicate *in vitro* resistance.

RESULTS

109 *C. albicans* strains, 16 *C. glabrata* strains, 10 *C. kefyr* strains, 6 *C. tropicalis* strains, 3 *C. sphaerica* strains, 3 *C. famata* strains and 2 *S. cerevisiae* strains were identified (Abaci et al., 2010). The result for the three quality control organisms were within the quoted reference ranges (Table 1). Amphotericin B, fluconazole, 5- fluorocytosine, caspofungin and terbinafine MIC ranges of *Candida* species and MIC_{50} and MIC_{90} values, whose susceptibility are evaluated in the study, are shown in Table 2. MIC_{50} : MIC at which 50% of the isolates were inhibited; MIC_{90} : MIC at which 90% of isolates were inhibited.

D SCUSS ON

The most effective agents used in the treatment of *Candida* species are the antifungal agents belonging to polyene and azole groups. Nystatin, amphotericin B, myconazole, fluconazole, itraconazole are generally used in the management of prostheses stomatitis (Farah et al., 2000; Akpan and Morgan, 2002). Although there are several treatment alternatives, widespread administration of antifungal agents has caused fungal pathogens resistant to one or more agents to emerge. In order to avoid problems regarding resistant fungi and to develop prophylactic and therapeutic strategies, it is vital to understand the nature of antifungal drug resistance (Rogers, 2006).

Fluconazole is a triazole agent that is established as a first-line antifungal for the treatment of oral candidiasis (Casalınuovo et al., 2004). Although isolates of *C. albicans* have been found to be susceptible to

Table 1. Quality control results for *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019 and *C.krusei* ATCC 6258.

Antifungal	*C. albicans* ATCC 90028		*C. parapsilosis* ATCC 22019		*C. krusei* ATCC 6258	
	Reference range (µg/ml)	Result (µg/ml)	Reference range (µg/ml)	Result (µg/ml)	Reference range (µg/ml)	Result (µg/ml)
Amphotericin B	0.5-2	1	0.5-4	1	1-4	2
Fluconazole	0.25-1.0	1	1-4	4	16-128	32
5-fluorocytosine	0.5-2.0	0.5	0.12-0.5	0.25	8-32	8
Caspofungin	-	2	0.5-4.0	2	0.25-1	0.5
Terbinafine (Ryder et al., 1998)	1	2	0.03	0.03	>128	128

Table 2. Minimum inhibitory concentration and MIC50 and 90 values obtained for amphotericin B, caspofungin, fluconazole, 5-fluorocytosine and terbinafine for *Candida* spp. by the CLSI methods.

Candida spp. (no. tested and antifungal agent	Range	Susceptible (%)	Susceptible dose dependent /intermediate (%)	Resistant (%)	MIC 50	MIC 90
***C. albicans* (109)**						
Amphotericin B	0.25-4	61 (56)	37 (33.9%)	11 (10.1)	1	4
Caspofungin	0.0625-4	108 (99.01)		1 (0.9)	0.25	0.25
Fluconazole	0.5-64	44 (40.4)		65 (59.6)	64	64
5- fluorocytosine	0.125-8	109 (100)			0.25	0.5
Terbinafine	32 and 128			109 (100)	128	128
***C. glabrata* (16)**						
Amphotericin B	2 -4		10 (62.25)	6 (37.5)	2	4
Caspofungin	0.125-2	16 (100)			0.25	0.5
Fluconazole	0.5-64	9 (56.25)	5 (31.25)	2 (12.5)	4	64
5-fluorocytosine	0.125-0.25	16 (100)			0.125	1
Terbinafine	128			16 (100)	128	128
***C. kefyr* (8)**						
Amphotericin B	0.5-4	3 (37.5)	3 (37.5)	2 (25)	2	-
Caspofungin	0.0625-0.25	8 (100)			0.125	-
Fluconazole	1-16	6 (75)	2 (25)		1	-
5-fluorocytosine	0.5-8	7 (87.5)	1 (12.5)		2	-
Terbinafine	128			8 (100)	128	-
***C. tropicalis* (5)**						
Amphotericin B	2 and 4			5 (100)	4	-
Caspofungin	0.5	5 (100)			0.5	-
Fluconazole	4		5 (100)		4	-
5-fluorocytosine	0. 25	5 (100)			0.25	-
Terbinafine	128			5 (100)	128	-
***C. krusei* (4)**						
Amphotericin B	4			4 (100)	4	-
Caspofungin	0.125	4 (100)			1	-
Fluconazole	0.5	2 (50)		2 (50)	0.5	-
5-fluorocytosine	8	2 (50)	2 (50)		4	-
Terbinafine	1-128	2 (50)		2 (50)	0.125	-

fluconazole (Pfaller et al., 2003), recently an increase in the isolation of azole-resistant *C. albicans* strains has been reported (Waltimo et al., 2000). In the present study, 59.4% of *C. albicans* (65/109) was found to be resistant

Table 2. Contd.

***C. sphaerica* (3)**						
Amphotericin B	2			3 (100)	-	-
Caspofungin	0.0625	3 (100)			-	-
Fluconazole	0.5	3 (100)			-	-
5-fluorocytosine	2	3 (100)			-	-
Terbinafine	128			3 (100)	-	-
***C. famata* (2)**						
Amphotericin B	2		2 (100)		-	-
Caspofungin	0.0625	2 (100)			-	-
Fluconazole	0.5	2 (100)			-	-
5-fluorocytosine	2	2 (100)			-	-
Terbinafine	128			2 (100)	-	-

to fluconazole (64 µg/ml). *C. albicans* molecular resistance mechanisms to azoles include: changes in the candidal target enzyme (lanosterol 14- demethylase), coded by *ERG11* gene; expression of transporter proteins which function as efflux pumps (ABC), coded by *CDR1* and *CDR2* genes; and major facilitators genes (*MDR1*). It has been shown that *CDR1* expression can be induced by azoles and 5- fluorocytosine (Casalınuovo et al., 2004; Richter et al., 2005). Most non- albicans *Candida* species have higher MICs to azole antifungal agents, and infections they cause are often difficult to treat. According to the results we obtained, all of the *C. famata* strains (2/2) were susceptible dependent upon dose; of the *C. glabrata* strains, 56.25% (9/16) were sensitive, 31.25% (5/16) were susceptible dependent upon dose and 12.5% (2/16) were resistant; of the *C. kefyr* strains, 25% (2/8) were susceptible dependent upon dose and 75% (6/8) were sensitive; of the *C. krusei* strains 50% (2/4) were sensitive and 50% (2/4) were resistant; all of the *C. sphaerica* (2/2) were sensitive; and all of the *C. tropicalis* strains (5/5) were susceptible dependent upon dose to fluconazole. Fluconazole has also been shown to be effective in treatment of palatal candidosis. Fluconazole also inhibit the adhesion of *Candida* to epithelial cells. Since fluconazole is secreted in high concentrations in saliva it may help reduce candidal colonization by interact with structure of *Candida* receptors on buccal epithelial cells (Ellepola and Samaranayake, 2000).

Amphotericin B is the most commonly used polyene antifungal. It has been in use since the 1950s. It has a broad spectrum of activity. There have only been few reports on resistant *C. albicans* isolates. In our study, 56% (n=61) of *C. albicans* was found to be susceptible to amphotericin B. Recently there have been reports on resistant *C. glabrata* and *C. krusei* isolates. Resistant isolates have also been found in *C. tropicalis*, *C. parapsilosis*, and *C. lusitaniae*. *C. glabrata* is considered as intermediate or susceptible dependent upon dose (Ellis, 2002). Consistent with these data, we found that, of the *C. glabrata* strains we isolated, 37.5% (6/16) were resistant and 62.25% (10/16) were susceptible dependent upon dose (10/16), and all the *C. krusei* strains (4/4) we isolated were resistant to amphotericin B. We also found that all of the *C. famata* strains (2/2) were susceptible dependent upon dose; of the *C. kefyr* strains, 37.5% (3/8) were sensitive, 37.5 (3/8) were susceptible dependent upon dose and 25% (2/8) were resistant; and all of the *C. sphaerica* strains (3/3) were resistant to amphotericin B. Of the 512 *C. albicans* (Kuriyama et al., 2005) were isolated from oral candidiasis patients, only 0.3% were resistant to fluconazole. They also found very low resistance rate in C*. glabrata* (5/59), *C. krusei* (0/7) and *C. parapsilosis* (0/12) strains. The highest resistance was seen in *C. krusei* strains (14.3%).

It was also determined that amphotericin B prevents *C. albicans* adhesion to buccal epithelial cells and germ tube formation in lower concentrations. In addition, dental adhesion of denture acrylic treated with amphotericin B is also greatly prevented. The former effects may be due to the mechanism of action of amphotericin B on the candidal cell wall, while the latter may be due to the blocking of the yeast attachment sites on the denture acrylic by the drug. Subminimal inhibitory concentrations (sub-MIC) of amphotericin B reduce proteinases activity of oral *C. albicans* isolates (Ellepola and Samaranayake, 2000).

Fluconazole and amphotericin B are frequently used in the treatment of oral candidiasis (Rogers, 2006). However, amphotericin B have some undesirable side-effects, such as significant renal toxicity, while prophylactic exposure to fluconazole can lead to resistance or overgrowth by naturally resistant organisms like *C. krusei* and *C. glabrata*. The limitations have led to a search for more effective antifungals. Caspofungin is a echinocandin and act by inhibiting of the synthesis of 1,3-ß-D-glucan in the fungal cell wall. Caspofungin is as effective as conventional amphotericin B for treating mucosal and systemic candidiasis (Cannon et al., 1995; Nicholas et al., 2004; Chenn and Sorrell, 2007). It is

known that caspofungin is as effective as fluconazole in the treatment of thrush and esophagitis. It was determined that fluconazole-resistant *C. albicans* isolates collected from esophagitis patients were sensitive to caspofungin (Hernandez et al., 2004). It was observed that the treatment of azole-resistant oral-esophageal candidiasis cases seen in AIDS patients with caspofungin was successful (Jokela and Kaur, 2007). In our study only 1 (0.9%) of 109 *C. albicans* strains was resistant to caspofungin. We also found that all of the non-albicans *Candida* strains were susceptible.

Terbinafine hydrochloride [(Ter-HCl), (E)-N-(6,6-dimethyl-2-hepten-4-yn-yl) -N-methyl-1-naphthalenemethanamide hydrochloride], is a new potent antifungal agent of the allylamine class which selectively inhibits fungal squalene epoxidase. It has a broad-spectrum activity against yeast, fungi, molds (eg. *Aspergillus* and *Penicillium* species) and dermatophytes and is indicated for both oral and topical treatment of mycoses (Özcan et al., 2009). Ryder and coworkers indicates that terbinafine has good activity against at least some azole-resistant *C. albicans* strains. Using 80% inhibition of growth as the assay end point, clear and reproducible MICs were obtained of terbinafine for the *C. albicans*. In our study, all *Candida* isolates were determined as resistant to terbinafine, except 2 *C. krusei* strains were susceptible. In accordance with our study, Figueiredo et al. (2007) determined that terbinafine presented low activity for strains of *C. albicans* and *C. tropicalis* isolated from fingernail infection. It is thought that *CDR1*, *CDR2* and *BEN* ^{r} which are responsible for fluconazole resistance may all impact resistance to terbinafine. *CDR1* can use terbinafine as a substrate (Ghannoum and Rice, 1999).

5- fluorocytosine is rarely used in the management of oral candidiasis. The DNA analogue 5-fluorocytosine is a fungistatic agent which is highly effective against all *Candida* spp. In a study conducted by Blignaut et al. (2002) in order to determine the susceptibility of 589 *Candida* strains they collected from HIV/AIDS patients' and healthy individuals' mouths to antifungal substances, they found that only 2.3% of *C. albicans* strains were resistant to 5FC. In our study, all *C. albicans* strains were sensitive to 5-fluorocytosine. Of all the non-albicans *Candida* strains, only 2 *C. krusei* strains and 1 *C. kefyr* strain were found to be susceptible dependent upon dose. 5 -fluorocytosine transported into the fungal cell by the action of cytosine permease, and inside the cell, is converted to form the active metabolite 5-fluorouracil by cytosine deaminase. 5-fluorouracil is then incorporated into RNA in place of uracil, with resulting abnormalities of protein synthesis. In addition, it blocks thymidylate synthetase, causing inhibition of DNA synthesis. Resistance to 5- fluorocytosine may result from decreased uptake or loss of enzymatic activity responsible for conversion to 5- fluorouridylic acid (FUMP). Since the mammalian cells lack cytosine deaminase, they are not affected by the drug (Ghannoum, 1999).

It is known that different oral infections such as hyperplasic candidiasis (CHC) can develop in denture wearers. CHC development is especially of importance in terms of malignancy development in that area. Because, *C. albicans* induces neoplastic developments by inducting the production of carcinogenic nitrosamines in the saliva (Sitheeque and Samaranayake, 2003; Hadjieva et al., 2006). It is seen that while some of the isolates we collected were resistant to antifungal agents such as amphotericin B and fluconazole which are frequently used in the treatment of *Candida*-associated denture stomatitis, the isolates we collected were susceptible to caspofungin and 5 -fluorocytosine which are rarely used in the treatment of oral fungal infections. Thus, it was concluded that these antifungal agents could be used as alternative drugs in oral infections which develop due to strains especially due to strains resistant to amphotericin B and fluconazole.

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