Antifungal susceptibility of *Candida albicans* isolated from the oral cavities of patients with HIV infection and cancer

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ABSTRACT

This study investigated the antifungal susceptibility of *Candida albicans* isolated from the oral cavities of 205 HIV positive patients, 49 cancer patients and 20 normal healthy individuals. *C. albicans* were isolated and the antifungal susceptibility was determined. The results were analysed using the clinical break points and epidemiological cut off values. Prevalence of *C. albicans* carriage in HIV, in cancer patients, and in healthy individuals was 73%, 45% and 43% respectively. Resistance of the fungus to anidulafungin (0.49%), caspofungin (0.97%), posaconazole (3.4%), voriconazole (0.97%), itraconazole (0.97%), fluconazole (1.94%), amphotericin B (0%) was found to be low. For posaconazole the number of resistant strains and the non-wild type (3.4%) were the same. However for the rest of the antifungal drugs, the number of non-wild type was found to be higher than the resistance determined by clinical break points. Multi-azole resistance was also noted in some patients. In conclusion, there is a low rate of antifungal drug resistance among *C. albicans* isolated from the oral cavities of immunocompromised patients in Johannesburg, South Africa. However, the high number of non-wild type strains suggests that there is a need for an ongoing surveillance.

ACRONYMS

ECV:	Epidemiological Cutoff Values
CBP:	Clinical Break Point
CLSI:	Clinical Laboratory Standards Institute
EUCAST:	European Committee on Antimicrobial Susceptibility Testing
MIC:	Minimal Inhibitory Concentration

INTRODUCTION

Candida albicans is a commensal of the human oral cavity, gut and vagina. This yeast causes infections in immunocompromised individuals including HIV and cancer patients. Oral candidiasis is the most common infection in these patients. It is frequently treated with antifungal agents such as amphotericin B, nystatin and fluconazole. In addition, prophylactic antifungals, particularly fluconazole, are prescribed to many patients undergoing cancer therapy especially in advanced cases and in patients with HIV infection (Epstein, 1996, Greenspan, 1994).[1,2]

Prior to the introduction of HAART, recurrent oral candidiasis was a problem in HIV positive patients, and still occurs, although to a lesser extent, in the era of HAART therapy. The development of antifungal resistance has been related to the use of antifungal agents to treat recurrent infections in patients with HIV where the appropriate doses, prescribed for the usual duration, become ineffective.[3] *Candida* species have exhibited a very high level of variability in the pattern of sensitivity to antifungal agents.[4] In Africa, routine antifungal susceptibility testing is not undertaken because oral thrush is treated with empirical antifungal agents. Consequently, the data on the antifungal sensitivity of *Candida* species from South Africa is still very sparse. A large study on the antifungal sensitivity profile of oral isolates was published more than 10 years ago, before fluconazole became widely available.[5] More recently, antifungal testing has become standardized globally through the Clinical Laboratory Standards Institute (CLSI) with the objective of detecting sensitivity patterns and discovering the development of resistant strains which is a growing problem.[2] It is therefore important to characterize the pattern of antifungal susceptibility of *Candida* species in the era of moderate availability of antifungal agents. This knowledge serves as a guide for antifungal therapy and can also help to predict the outcomes of therapeutic interventions. The present study investigated the antifungal sensitivity profile of *C. albicans* isolated from the oral cavities of HIV positive patients, cancer patients on chemotherapy or radiotherapy and normal healthy individuals.

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MATERIALS AND METHODS

Study population and identification of *C. albicans*

Ethics clearance was obtained from the Committee for Research on Human Subjects (Medical), University of the Witwatersrand, Johannesburg, Gauteng. One hundred and nine patients, diagnosed with mainly head and neck cancers, who were scheduled for either radiation or chemotherapy and were attending clinics at the Department of Oncology, together with 529 HIV positive patients who were attending the HIV clinic in Charlotte Maxeke Johannesburg Academic Hospital, were asked to volunteer for the study. Forty nine normal healthy individuals without any signs of oral candidiasis were also included in the study since *Candida* is a commensal in oral cavities and candidiasis does sometimes develop. All these patients were also the subjects of three other studies.

The procedure was explained to participants and written consent was obtained. Data such as the presence of active infection, other risk factors and previous exposure to antifungal agents were not available due to incomplete patient records and a lack of verification by the patients. Heterogeneity of the study population and the incomplete patient data are limitations of this study.

An oral rinse with 10ml of sterile distilled water was used to collect samples in sterile sputum jars. 100 µl of the rinse sample was inoculated onto CHROMagar® *Candida* plates (CHROMagar Microbiology) and incubated at 37°C for 48 hours. CHROMagar is a chromogenic agar that supports the growth of many *Candida* species and even enables the identification of some species on the basis of the colours of the colonies. For example, *C. albicans* presents green colonies, *C. tropicalis* produces steel blue colonies and *C. krusei*, purple colonies. All the different colour colonies were selected, subcultured on Sabouraud dextrose agar for 48 hours for purity and identified using API 20 C AUX system® (bioMérieux). This is a standard substrate assimilation test that can accurately identify *Candida* species. These cultures were stored in microbank vials at -70°C for further studies.

Determination of antifungal susceptibility

Financial constraints limited testing for antifungal susceptibility to only 205, 49 and 20 strains of *C. albicans* which had been isolated from, respectively, HIV patients, cancer patients, and normal healthy individuals. The tests were performed using Sensititre YeastOne® microdilution colorometric microtitre plates. The procedure is based on the Broth Microdilution minimum inhibitory concentration system described by Clinical Laboratory Standards Institute document M27-A2.[6] The microtitre plates contained two-fold dilutions of anidulafungin, micafungin, caspofungin, 5-flucytosine, posaconazole, voriconazole, itraconazole, fluconazole, amphotericin B. Also contained was AlamarBlue®, a proven cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin, which produces very bright red fluorescence. Fresh yeast cultures (five randomly selected colonies) were suspended in normal saline and adjusted to a turbidity of 0.5 McFarland standards. 20µl of the inoculum was added to the Sensititre YeastOne® broth and gently vortexed. 100 µl of the inoculated broth was added to each well of the Sensititre YeastOne® panel using a multichannel pipette and incubated at 35°C for 24 hours. Wells with growth were red in colour. The results were read and the minimal inhibitory concentration (MIC) was determined as the lowest concentration that prevented a growth (the first blue well). Control strains of *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included as recommended by the CLSI guidelines. The results were interpreted as clinical break point (CBP) and epidemiological cutoff values (ECV). Clinical break point represents clinical isolates that are likely to respond to treatment with a given antimicrobial agent administered using the approved dosing regimen for that agent. They are relevant to clinicians. By contrast, epidemiological cutoff values represent the most sensitive measure of the emergence of strains with decreased susceptibility to a given agent. This means that ECV results will detect development of resistance ahead of clinically relevant resistance, which would present as a treatment failure due to drug resistance. The re-established clinical break points and epidemiological cutoff values of the CLSI and the EUCAST for anidulafungin, micafungin, caspofungin, voriconazole, itraconazole and fluconazole as described by Pfaller and Diekema (2012),[4] and for amphotericin B and posaconazole as described by Arendrup *et al*. (2011)[7] and Lass-Flörl *et al*. (2011)[8] were used to characterize the sensitivity profiles of the *C. albicans* isolates.

Table 1: Prevalence of *Candida albicans* in the oral cavities of patients with HIV and cancer, and normal healthy individuals.

Study population	Total No. of patients	*Candida albicans* carriers No. of patients (%)	Predominant non-albicans *Candida*
HIV	529	387 (73.16)	*Candida dubliniensis* *Candida glabrata* *Candida parapsilosis* *Candida tropicalis* *Candida famata*
Cancer	109	49 (44.95)	*Candida glabrata* *Candida parapsilosis* *Candida kefyr*
Normal healthy	49	21 (42.86)	None

RESULTS

Prevalence of *C. albicans*

The carriage rate of *C. albicans* was 73.16%, 44.95% and 42.86% in patients with HIV, with cancer, and in normal healthy individuals, respectively (Table 1). Patients with HIV and cancer carried a variety of *Candida* species other than *C. albicans* in their oral cavities.

Antifungal susceptibility of *Candida albicans*

The range of MIC values (MIC obtained by all the strains), MIC50 (MIC obtained by 50% of the strains) and MIC90 (MIC obtained by 90% of the strains) obtained for the test antifungal agents are shown in Table 2. The interpretation of these MIC results were obtained using previously described standards.4,7,8 The characterized sensitivity profile of the *C. albicans* isolates are given in Table 3.

DISCUSSION

Candida albicans is the most frequently isolated *Candida* species from the oral cavities of HIV patients (73%) and cancer patients (45%) which is similar to elsewhere.[9,10] Although HAART has reduced the carrier rate of *Candida* species in these patients, the proportions of *C. albicans* and non-albicans *Candida* have not dramatically changed.[9] Therefore the antifungal agents are still used to target *C.*

Table 2: MIC results of *Candida albicans* isolated from the oral cavities of HIV and cancer patients, and normal healthy individuals.

Antifungals	MIC (µg/ml) n=275		
	MIC range	MIC_{50}	MIC_{90}
Anidulafungin	<0.015 - 8	0.03	<0.015 - 0.06
Micagungin	< 0.008 - 8	< 0.08	< 0.008 - 0.08
Caspofungin	0.015 - 8	0.15	*0.015 - 0.06*
5-Flucytosine	< 0.06 - > 64	0.06	< 0.06 – 0.06
Posaconazole	< 0.008 – 8	0.008	< 0.008 – 0.03
Voriconazole	< 0.008 – 8	< 0.008	< 0.008 – 0.008
Itraconazole	< 0.015 – 16	0.015	*0.015 – 0.06*
Fluconazole	< 0.12 – 128	0.12	0.12 - 0.5
Amphotericin B	< 0.12 – 0.5	0.25	0.12 – 0.25

Minimum Inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism.

MIC_{50} and MIC_{90} are concentrations that respectively inhibit 50% and 90% of *C. albicans* isolates.

albicans and the sensitivity profile of *C. albicans* has been most commonly described. However, due to the overuse and frequent use of antifungal drugs, resistance to these drugs has increased.[11] Although fluconazole was freely available in sub-Saharan Africa, results in this study show that the resistance to this drug is very low i.e. 1.94% (Table 2 and 3) because it is still a prescription drug in South Africa. This resistance has slightly increased since a zero resistance was reported in 2002.[5] In Tanzania, where 250 isolates of *C. albicans* were tested, there was no resistance to fluconazole whereas 4% of isolates were resistant to itraconazole and 0.54% azole resistance has been reported from Tunisia.[12,13] More recently, 50% resistance of *C. albicans* to fluconazole has been reported in southern parts of South Africa.[14] Antifungal drug resistance varies in the several African countries. A study in Nigeria in 2011[15] and recently from Ethiopia[16] reported 16% resistance to fluconazole in *C. albicans* whereas a resistance among isolates from Cameroon was as high as 50%.[14]

Less than 1% of strains that were tested in this study were resistant to the echinocandins i.e. anidulafungin, micafungin, and caspofungin (Table 3). This is consistent with other reports that ranged from of 1% to 5%.[16-18] The reason may be that these drugs have only recently become available in Africa.

Seven isolates (3.4%) of *C. albicans* were found to be resistant to posaconazole which is unexpected (Table 2). It is the most recently approved triazole with a broad spectrum activity against *Candida*, *Aspergillus*, *Cryptococcus* and many other fungi and is usually reserved for the treatment and prophylaxis of invasive forms of fungal infections. The breakpoints for posaconazole are very close to one another and they are not well described (S < 0.06 and R >0.06). These values are based on pharmacokinetic data epidemiological cut-off values and clinical experience and therefore they are reviewed regularly.[7] However, in our study for posaconazole the number of resistant strains and the non-wild type (3.4%) were identical, showing good correlation between the two methods of analysis. This also means that these non-wild type isolates may have acquired or innate mutational resistance mechanism and therefore may or may not show a clinical response to the antifungal therapy. In addition, during surveillance these epidemiological cut-off values are useful in detecting the emergence of potential resistance. This phenomenon has been recorded by Pfaller *et al.* (2011) whose study recorded that the epidemiological cut-off values for posaconazole increased three times from 2001 to 2009 (1.1 to 3.2%).[19]

Although a previous South African study reported that 8.4% of *C. albicans* were resistant to amphotericin B,[5] no resistance was detected in the present study. Furthermore, it is known that patients previously exposed to fluconazole for a long time period harbour more fluconazole-resistant *C. albicans* than patients who are fluconazole-naïve.[10,20] Unfortunately it was not possible to collect data on the history of previous antifungal exposure in all the HIV patients.

Table 3: Antifungal susceptibility of *Candida albicans* isolated from the oral cavities of HIV and cancer patients, and normal healthy individuals.

Antifungal agent	*Candida albicans* Isolates	Clinical break point				Epidemiological cutoff values	
		S	SDD	I	R (%)	WT	Non-WT
Anidulafungin	HIV	205	0	0	1 (0.49)	205	1
	Cancer	49	0	0	0	49	0
	Normal	20	0	0	0	20	0
Micafungin	HIV	205	0	0	0	204	2
	Cancer	49	0	0	0	49	0
	Normal	20	0	0	0	20	0
Caspofungin	HIV	204	0	0	2 (0.97)	204	2
	Cancer	49	0	0	0	49	0
	Normal	20	0	0	0	20	0
5-Flucytosin	HIV	ND	ND	ND	ND	194	12
	Cancer	ND	ND	ND	ND	43	6
	Normal	ND	ND	ND	ND	17	3
Posaconazole	HIV	199	0	0	7 (3.4)	199	7
	Cancer	49	0	0	0	49	0
	Normal	20	0	0	0	20	0
Voriconazole	HIV	203	0	1	2 (0.97)	201	5
	Cancer	40	0	0	0	49	0
	Normal	20	0	0	0	20	0
Itraconazole	HIV	202	2	0	2 (0.97)	202	4
	Cancer	49	0	0	0	49	0
	Normal	20	0	0	0	20	0
Fluconazole	HIV	202	0	0	4 (1.94)	198	8
	Cancer	49	0	0	0	48	1
	Normal	20	0	0	0	19	1
Amphotericin B	HIV	206	0	0	0	206	0
	Cancer	49	0	0	0	49	0
	Normal	20	0	0	0	20	0

S-sensitive, **SDD**-sensitive dose dependent, **I**-intermediate, **R**-resistant, **WT**-wildtype, **Non-WT**-non wild type, **ND**-not yet defined. **Wild-type strains:** Those without mutational or acquired resistance mechanisms (sensitive) **Non wild-type strains:** Those having mutational or acquired resistance mechanisms (possibly resistant)

However, that history was available for the cancer patients and the normal individuals. No antifungal drug resistance was found in the *C. albicans* isolates from the oral cavities of cancer patients or individuals in the healthy individuals. They had not been exposed to antifungal drugs prior to the respective collection of samples. In addition there was no prophylactic prescription of antifungal agents for patients undergoing either radiation therapy or chemotherapy in the Oncology Departments in this study.

Four fluconazole resistant strains of *C. albicans* were isolated from HIV positive patients. Two isolates were resistant to all four azole drugs whereas the remaining two isolates were either intermediate or dose dependent on two azole drugs but were resistant to fluconazole. Prophylactic itraconazole is known to induce resistance to fluconazole.[21] In addition, the use of ketoconazole and miconazole reduces the susceptibility to fluconazole.[22,23] Multiple-resistance seen in our study may have been due to cross resistance among azole drugs.

Strains categorized as resistant according to the clinical break points, are associated with a high likelihood of therapeutic failure. Non-wild type strains have acquired or innate mutational resistance mechanisms and therefore these strains may or may not show a clinical response to the antifungal therapy. In our results all the antifungal drugs except for posaconazole showed high epidemiological cut-off values compared with the clinical break points. This means that in our study population drug resistance is developing which will require regular studies or continuous surveillance.

These results have shown that resistance of *C. albicans* to commonly used antifungal agents is low. However, *C. albicans* resistant to one azole is likely to be resistant to other azole drugs. Therefore, patients who do not respond to an azole drug should be given drugs other than azole. Furthermore, pathological material should be sent for a microbiological analysis including culturing for *Candida*. Presence of multiple *Candida* in these patients suggests a need for further research and data on antifungal susceptibility in these yeasts.

In conclusion, the rate of antifungal drug resistance among *C. albicans* isolated from the oral cavities of immunocompromised patients is low in Johannesburg, South Africa. Multiple azole resistance was noted in HIV positive patients. Therefore, alternative antifungal drugs should be considered in non-responsive patients. In addition, high ECV suggested the likelihood of development of drug resistance and therefore ongoing surveillance is needed.

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