

Full Length Research Paper

Sensitivity and lower protease activity of *Candida albicans* species isolated from Egyptian cancer patients after exposure to cytotoxic and/or radiotherapy treatment

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Oropharyngeal candidiasis is a common disease among cancer patients receiving chemo or radiotherapy which precede systemic candidemia, a life threatening infection. This study investigated the diversity and prevalence of different *Candida* species among Egyptian cancer patients, evaluated the sensitivity of *Candida albicans* to the frequently administered antifungal therapies and the effect of different radio and chemotherapeutic agents on its virulence. A total of 119 *Candida* spp. isolates were identified out of 399 clinical samples, of which 72 isolates were *C. albicans*, 15 were *Candida tropicalis*, 22 were *Candida krusei*, and 10 were *Candida glabrata*. 98.6% of the *C. albicans* isolates were sensitive to fluconazole; on the other hand, only 8.3% out of the tested isolates were sensitive to amphotericin B. No significant differences were observed in the ability of biofilm formation among *C. albicans* isolates exposed to chemo, radio or both therapies when compared with standard *C. albicans* ATCC 60193. Surprisingly, the protease activities in isolates obtained from cancer patients were significantly lower than that of the reference strain after exposure to chemo, radio or both therapies. Thus, it is concluded that radio and chemotherapies may not be in some cases a predisposing factor for the virulence of *C. albicans* strains.

Key words: Candidiasis, antifungal agents, virulence, radio and chemotherapy.

INTRODUCTION

Cancer is one of the most serious health problems faced by many individuals in the course of their lives and it is

usually associated with high incidence of microbial infections (Bodey, 1986). Diseases involving *Candida*

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species are common in cancer patients (Ramirez-Garcia et al., 2014). The penetration of this fungal species into the bloodstream and its dissemination, causing candidemia, is a life threatening infection, being responsible for 30 to 50% of mortality rates among cancer patients (Nucci and Marr, 2005).

Candida albicans is the main resident flora of the digestive mucosa and the genital area, identified in approximately 10 to 20% of healthy adults, followed by *Candida glabrata* and *Candida tropicalis* (Zadik et al., 2010). *C. albicans* is responsible for about 80% of oropharyngeal infections (Bensadoun et al., 2011). Oropharyngeal candidiasis (OPC) is common among patients undergoing intensive chemo or radiotherapy and is known to precede systemic infections (Al-Attas and Amro, 2010). It is a major cause of morbidity in cancer patients (Bodey, 1986). The development of OPC results from the imbalance between fungal virulence factors and host defenses. Several known virulence factors contribute to the pathogenicity of *C. albicans*, which include adherence to host tissues, phenotypic switching, dimorphism conversion, and enzymes that are integral to its pathogenesis (Calderone et al., 2000; Liu, 2001). Proteinases and phospholipase B are the main enzyme categories secreted by different *Candida* spp. (De Bernardis et al., 2001).

The common applied therapy, in case of candidiasis, is fluconazole, where its effectiveness is highly proven and superior to other treatments, such as nystatin and clotrimazole with a broad therapeutic range and little toxicity (Sheehan et al., 1999). However, increasing fluconazole resistance in cancer patients is reported (Tortorano et al., 2004). The mechanism of resistance mainly depends on either mutation or over expression of the *erg11* gene leading to reduced drug affinity for the target enzyme or increase in ergosterol synthesis, respectively (Lupetti et al., 2002; Maebashi et al., 2003). Caspofungin, is the first member of a new class of antifungal agents targeting the fungal cell wall. It showed high effectiveness in the treatment of candidiasis (Letscher-Bru and Herbrecht, 2003), but unfortunately, its high cost limits its use in Egypt.

Chemotherapy may lead to damage to the mucosal barrier that may result in epithelial atrophy and mucosal ulceration, which may be associated with increased adherence and invasion of *Candida* (Bensadoun et al., 2011). On the other hand, antineoplastic agents may negatively affect morphogenesis, fungal growth, and virulence of *Candida* spp. as reported in *in vitro* studies (Chen et al., 2011). Hence, the present study aimed at the identification of different *Candida* spp. causing OPC infections and evaluating the sensitivity of *C. albicans*, the most common infective species in cancer patients, to the frequently administered antifungal therapies and the effect of different chemotherapeutic agents on the virulence of *C. albicans*. These data are urged to be continuously updated in order to tailor treatment and

update prevention guidelines.

MATERIALS AND METHODS

Sample collection

A total of 399 samples were collected from different cancer patients of both sexes and from adults as well as infants. Patients were submitted in Kasr El-Einy, Center of Radiation, Oncology and Nuclear Medicine, Faculty of Medicine, Cairo University (NEMROCK). All cases were diagnosed by physicians at the hospital. Samples were collected from the patients' oral cavity using sterile sealed swabs. The work was carried in accordance with the code of ethics of the world association (declaration of Helsinki) for experiments involving humans. A written consent was signed by the studied subjects or their parents after full explanation of the study. The ethical approval was obtained from the medical ethics committee at the Faculty of Pharmacy, Cairo University. The clinical data collected included the personal data, age, residence, clinical diagnosis and predisposing factors, such as diabetes mellitus, pregnancy, use of antibiotics, previous surgical operations, the use of immunosuppressant drugs, exposure to radiation and the type of this radiation.

Identification and maintenance of isolates

All samples were streaked on Sabouraud Dextrose Agar, Oxoid, CM0041 (SDA, pH 6.5) and incubated for 48 h at 37°C. A total of 119 isolates of *Candida* spp. were identified out of the 399 samples by Gram stain microscopic examination. Further identification was done by isolating the 119 samples on surface of *Candida* Ident Agar media, modified (Biochemika, Fluka, Sigma Aldrich, 94382), and incubated for 48 h at 37°C. Each isolate represents a unique strain from a single patient. All isolates were scraped from the media, suspended in Brain Heart broth; equal volumes of 30% sterile glycerol were added and mixed evenly using a vortex. The mixtures were then distributed into sterile Eppendorf tubes and stored at -80°C for long time preservation (Prasad et al., 2010). Identified *C. albicans* samples were subjected to susceptibility, biofilm formation, and protease activity assays in comparison to a standard strain of *C. albicans*, ATCC 60193.

Susceptibility testing

Antibiotic susceptibility was tested by disc diffusion method described in the Clinical and Laboratory Standards Institute (CLSI). Fluconazole (10 and 25 µg) and amphotericin B (100 units) disks were obtained from Hi-media (India) and Oxoid (Sparks, Md., UK). The standard *C. albicans* ATCC 60193 and each isolated *C. albicans* strain were sub-cultured on Sabouraud Dextrose Agar (SDA) plates for 48 h at 37°C. Three to four colonies were transferred aseptically into 5 ml of Sabouraud Dextrose broth and incubated at 25 to 30°C for 8 h. The turbidity of the suspension was adjusted to 0.125 at $\lambda = 550$ nm which is equivalent to 0.5 Macfarland standard (approximately 1.5×10^8 CFU/ml). Plates filled with SDA to a depth of 4.0 mm were used. The SDA surface was inoculated by using a swab dipped in the prepared cell suspension of each isolate in addition to the reference strain, then the antibiotic discs were placed on the surface of the SDA plates. The plates were inverted and incubated at 25 to 30°C for 40 to 48 h. Subsequently, the plates were examined and the zone diameters were measured in mm. The test was repeated twice for each isolated strain and the average diameter was calculated.

Table 1. Distribution of *Candida* spp. according to age and sex of patients.

<i>Candida</i> spp.	Patients				Total	Percentage of <i>Candida</i> spp.
	Male		Female			
	Adult	Infant	Adult	Infant		
<i>C. albicans</i>	38	3	31	0	72	60.5
<i>C. tropicalis</i>	9	0	6	0	15	12.6
<i>C. krusei</i>	13	0	9	0	22	18.5
<i>C. glabrata</i>	5	0	5	0	10	8.4
Total	65	3	51	0	119	-
Percentage of patients	54.6	2.5	42.9	0	100	-

Infant: 0-12 years; Adult: Above 12 years.

Biofilm formation test

C. albicans samples were inoculated in yeast nitrogen base media (YNB) supplemented with 100 mM glucose and cultured overnight. The yeasts were then harvested, washed twice with phosphate buffer saline (PBS, pH 7.2, Ca⁺² and Mg free), suspended to 10⁷ cells/ml by adjusting the optical density of the suspension to 0.38 at 520 nm, and used immediately. A volume of 100 µl of standardized cell suspension (10⁷ cells/ml) of each sample was transferred into sterilized micro titer plate and incubated in a shaker at 75 rpm, for 1.5 h at 37°C to allow the yeast to adhere to the surfaces of the wells. The plate was washed; fresh media was added and incubated again at 37°C in a shaker at 75 rpm. The biofilms were allowed to develop up to 66 h and then the yeasts were quantified by the crystal violet assay as described earlier (Djordjevic et al., 2002).

Quantitative protease assay

Protease activity of *C. albicans* was assessed by a commercial kit (Pierce Quantitative Protease Assay Kit, ThermoScientific, 23263, Hyclone, USA) according to the method described in Rao et al. (1997) and modified by Tian et al. (2004). The inoculums were adjusted to 0.8 at 550 nm to get 10⁸ cells/ml as described by the manufacturer's protocol. The protease activity was measured using a plate reader at 450 nm.

Statistical analysis

Data of protease activity and biofilm formation experiments were expressed as mean ± standard error of means (SEM). Significant difference was calculated by one way analysis of variance (ANOVA) for both biofilm formation test and quantitative protease assay using Graphpad prism 5 for windows. Differences in results with p<0.05 were considered to be significant. The demographic distribution data were analyzed using Chi square test. The data were expressed as absolute count number. Significance at p value<0.05 was considered.

RESULTS

Demographic distribution of samples

Out of 399 screened patients, only 119 (29.8%) were confirmed to have OPC. The presented samples were

distributed according to sex to 57% males and 43% females, of which 29.4% were identified as smokers and 61.3% were non-smokers. Out of 119 samples, *C. albicans* represented 60.5% (72 isolates) of the total identified *Candida* isolates. The percentage of other isolates were 12.6% *C. tropicalis* (15 isolates), 18.5% *C. krusei* (22 isolates), and 8.4% *C. glabrata* (10 isolates) (Table 1).

Of the *C. albicans* identified patients, 32 received chemotherapy before sampling and 14 patients were on radiotherapy, while 24 patients received both chemo- and radio-regimens. Two patients did not receive either chemo or radiotherapies and were referred to as unknown treatment in the present study.

Figure 1 shows the incidence and prevalence of each *Candida* spp. among haematological and solid cancer types. It was observed that *C. albicans* is more prevalent in solid cancer types (64%) followed by *C. krusei*, then *C. glabrata* and finally *C. tropicalis*. Moreover, in the haematological cancer, *C. albicans* was also the most prevalent (53%), followed by *C. tropicalis*, *C. krusei* and *C. glabrata*. The prevalence of *C. albicans* and *C. krusei* was much higher in solid cancer types than in haematological cancer, while the opposite is true for *C. tropicalis* and *C. glabrata*.

Figure 2 shows the incidence and prevalence of each *Candida* spp. among haematological and solid cancer types in relation to different cancer treatments. It was observed that in chemotherapy, the haematological cancer type was more prevalent than the solid cancer type in all *Candida* spp. While in radiotherapy, only solid cancer type was observed in all *Candida* spp. except *C. glabrata* which was found neither in solid nor in haematological cancer types.

Susceptibility testing

For fluconazole (10 and 25 µg), 98.6% of the isolated *C. albicans* were sensitive, 1.4% were of intermediate susceptibility and none of the samples showed resistance. Trailing phenomena was observed and

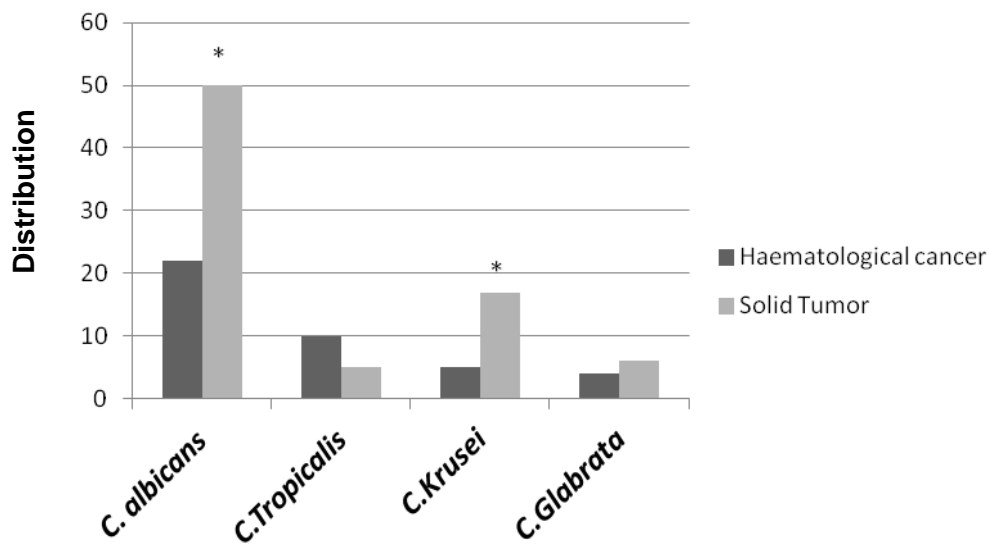


Figure 1. Distribution of *Candida* spp. among different cancer types. Significance is calculated using Chi square test P value<0.05.

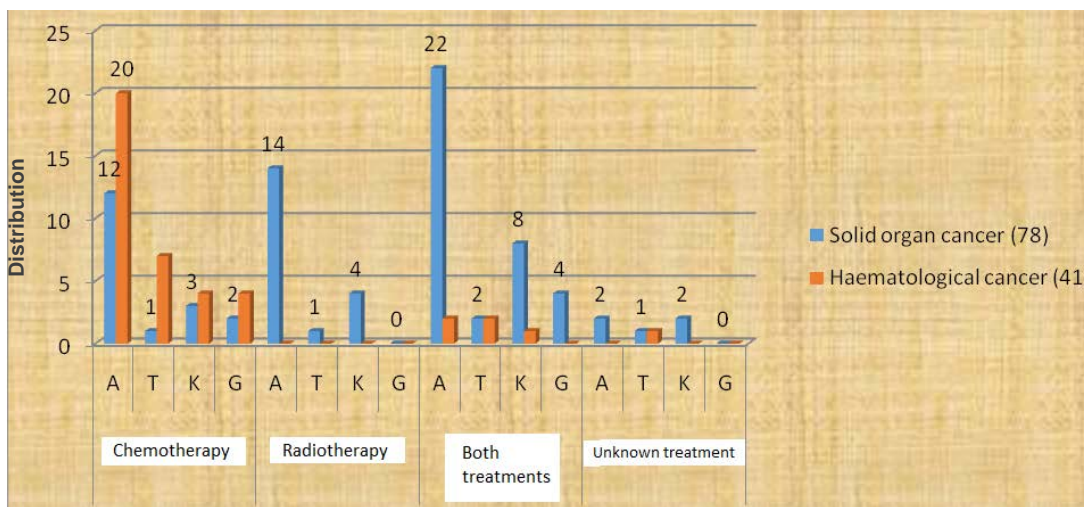


Figure 2. Distribution of *Candida* spp. among different types of cancer treatment and cancer types. Significance is calculated using Chi square test P value<0.05.

considered as sensitive results. For amphotericin B, 8.3% of the tested *C. albicans* isolates were sensitive, while 87.5% were of intermediate susceptibility and 4.2% were resistant. The standard ATCC 60193 was sensitive to fluconazole and of intermediate susceptibility to amphotericin B. The interpretation of the results was based on the criteria specified by the CLSI. All results are shown in Table 2.

Biofilm formation

The biofilm formation of *C. albicans* isolates from each of

the 4 groups of cancer patients was compared to that of the standard ATCC 60193. No significant differences were observed among chemotherapy (0.449±0.002), radiotherapy (0.450±0.003), both therapies (0.446±0.001), or unknown treatment (0.459±0.01) groups as compared to the standard ATCC 60193. Results are demonstrated as show in Figure 3.

Protease production

The protease concentrations in *C. albicans* isolates obtained from cancer patients were significantly lower

Table 2. Susceptibility testing of isolated *C. albicans* strains by disc diffusion method.

Antifungal agent	Disc content	Breakpoint (mm)			Resistant		Intermediate		Sensitive		ATTC
		R	I	S	Frequency	%	Frequency	%	Frequency	%	
Fluconazole (FU ¹⁰)	10 µg	≤18	19-21	≥22	0	0	1	1.4	71 (T)	98.6	T
Fluconazole (FCA)	25 µg	≤25	26-29	≥30	0	0	1	1.4	71 (T)	98.6	T
Amphotericin B (AP ¹⁰⁰)	100 Units	≤10	11-17	≥18	3	4.2	63	87.5	6	8.3	I

R: Resistant; S: Sensitive; I: Intermediate; T: Trailing.

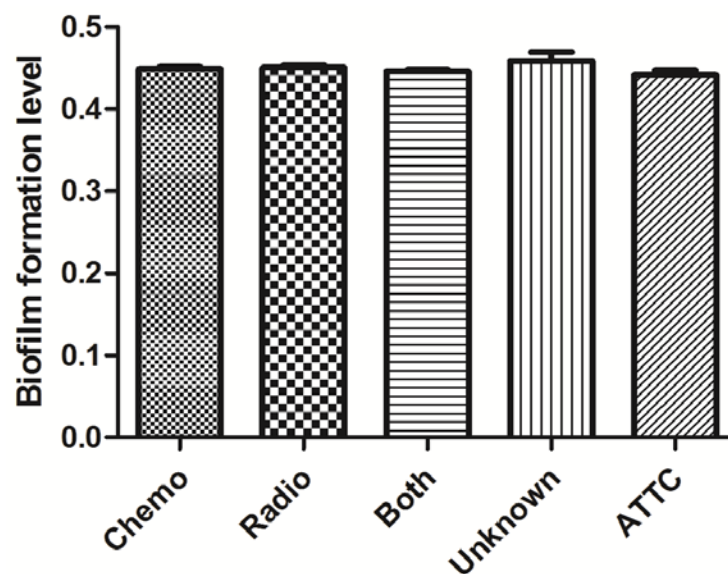


Figure 3. Biofilm formation of *C. albicans* among different therapy groups. Data represent mean \pm standard error of mean (SEM). Statistical analysis was carried out by one-way analysis of variance (ANOVA).

than that of *C. albicans* ATCC 60193 reference strain after exposure to chemotherapy, radiotherapy, both treatments, or unknown treatment (Table 3).

DISCUSSION

Patients who undergo chemo and/or radiotherapy are at increased risk of developing fungal

infection. In the case of changes in the mucous membrane, fungi can move into the blood and develop into disseminated fungal infection, often leading to death. Hence, it is important to determine

Table 3. Protease enzyme levels of *C. albicans* in cancer patients received chemotherapy, radiotherapy or both, compared to *C. albicans* ATCC 60193 reference strain.

Group	Protease level (ng/ml)	Number of samples
Chemotherapy	185.9±10.2*	20
Radiotherapy	204.2±17.1*	11
Both therapies	178.7±8.4*	18
ATCC 60193	249.3±0.8	1

Data represent mean ± standard error of mean (SEM). Statistical analysis was carried out by one-way analysis of variance (ANOVA). * $p < 0.05$ vs. ATCC 60193 standard sample.

the presence of fungi in this group of patients before the beginning of chemo- or radiotherapy to enable early treatment. The current study discussed the prevalence and the virulence of *C. albicans* among this group of patients. A total of 60.5% of candidiasis subjected in this study was identified as *C. albicans*, which is a large proportion despite the widespread use of fluconazole. On the other hand, other *Candida* spp. were found in minor proportions. This complies with a previous report, where *C. albicans* constituted 56.3% of patients receiving radiotherapy for head and neck neoplasms, while each of *C. glabrata* and *C. tropicalis* was present in 12.5% of the total patients (Tudela et al., 2002). Furthermore, most of the OPC samples from cancer patients in different regions were also identified as *C. albicans* with a range of 33 to 76% of the total isolates (Laverdiere et al., 2002; Al-Abeid et al., 2004; Belazi et al., 2004).

This study also showed higher prevalence of *C. albicans* in both solid and haematological cancer types, followed by *C. krusei*, then *C. glabrata* and finally, *C. tropicalis* in solid cancer types. While in the haematological cancer types, *C. albicans* was found to be followed by *C. tropicalis*, *C. krusei* and *C. glabrata*, respectively. The prevalence of *C. albicans* and *C. krusei* was much higher in solid cancer types than in haematological cancer, while the opposite is true for *C. tropicalis* and *C. glabrata*. This was also the case in a study made by Slavin et al. (2010) where the prevalence of *C. albicans* in solid organ malignancies was higher than in haematological malignancies, 51 and 33%, respectively.

The present study showed that chemotherapy discloses the most incidence of *C. albicans* infection (44%), followed by the combined chemo-radiotherapy, a result that coincides with the study made by Al-Abeid et al. (2004) where the chemotherapy patients were colonized more frequently by *C. albicans* than radiotherapy patients.

The adherence level of the studied isolates was found not to be significantly different from that of ATCC control strain. This result does not agree with that obtained from the study made by Al-Abeid et al. (2004), where there was a statistically significant difference between the

number of adhered *C. albicans* and the ATCC control strain.

Our findings also revealed that the protease production was lowered in isolates from different cancer patients than that of ATCC control strain. The same result was suggested by Al-Abeid et al. (2004). This may be explained by the fact that, some cytotoxic agents can lower the protease production in *C. albicans* as various anticancer agents, such as glucocorticoids, cytotoxic agents and calcineurin inhibitors have direct inhibitory effects on and/or have altered the biology of fungal cells and in some cases can also be used as a combined therapy with antifungal agents (Chen et al., 2011). Although the virulence of the *C. albicans* is lowered, the infection and colonization persists in cancer patients, this may be due to the immune-suppression effect of anticancer treatments.

Disk diffusion method was used to determine the susceptibility of *C. albicans* to fluconazole and amphotericin B as it represents a reliable and reproducible method (Rodríguez-Tudela et al., 1996). *C. albicans* isolates in the current investigation were susceptible to fluconazole and of intermediate sensitivity to amphotericin B suggesting the prime role the fluconazole has in fungicidal activity. These results are in accordance with other studies reporting susceptibility of *C. albicans* in cancer patients (Al-Abeid et al., 2004; Slavin et al., 2010). Additionally, it was noted that strains exhibiting trailing growth, as seen in the present experiment, responds to low doses of fluconazole (Revankar et al., 1998).

In conclusion, the effective management of *C. albicans* infections prior to any anti-cancer treatment is highly recommended in order to avoid the complications that might arise from the fungal infection upon starting the anticancer treatment. Although the current study indicates that cancer therapy, either chemotherapy or radiotherapy, has affected the virulence of *C. albicans*, it is highly recommended to keep caution when dealing with powerful antifungal agents in the prophylaxis or the management of OPC especially in immune-compromised patients to avoid the possible emergence of resistant strains.

Conflict of Interests

The authors have not declared any conflict of interests.

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