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Genotyping and Antifungal Susceptibility Profile of *Candida albicans* Isolated from Cancer Patients

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ABSTRACT

The aim of this study was to determine the genotypic and antifungal susceptibility of *C. albicans* obtained from cancer patients. The study included fifty cancer patients treated with chemotherapy who exhibited evident oral lesions. Oral swabs from cancer patients and healthy persons screened for the occurrence of *C. albicans*. Isolates were identified by the conventional mycological methods. Genotypes were determined using 25S rDNA PCR analysis. Oral *C. albicans* was detected in 84.0% cancer patients and 52.0% healthy persons. PCR targeting 25S rDNA genotype analyses of *C. albicans* obtained from cancer patients, allowed isolates to be grouped into genotypes A, B, C and T, among which genotype A *C. albicans* constitutes the majority of this fungus. Genotype A *C. albicans* recognized the entirely isolates in healthy group. Isolates were most sensitive to amphotericin B. Isolates has shown high rate of resistance to fluconazole and ketoconazole. 25S rDNA have been shown to be a useful criterion for distinguishing among various isolates of *C. albicans*. Amphotericin B is effective antifungal agents that can be used against isolates. The study believes that description of active pathogens in *Candida* infections at the genotype level and research on antifungal sensitivity will be very useful in epidemiology, managing treatment, and preventing resistance development in hospitals.

KEY WORDS: *Candida albicans*, 25S rDNA, Cancer patients, Genotyping.

1. INTRODUCTION

Candida species are part of normal microflora of oral cavity in 40% to 60% of healthy individuals. There has been an increase in the frequency of diseases caused by *Candida* over the recent past. The majority of these diseases are caused by *C. albicans* (McCullough, 1996; Fidel and Wozniak, 2005) *C. albicans* is the most common cause of oral candidosis in cancer patients receiving radiotherapy and/or chemotherapy (Lalla, 2004).

Rapid identification of the etiological agent is important for the success of treatment so that molecular techniques are increasingly used to shorten identification time (De Baere, 2002). Molecular typing of an infectious agent is important for epidemiological studies and for the development of suitable infection control strategies (McCullough, 1999). To understand the epidemiology of *C. albicans* infection, various molecular techniques have been used for strain description (McCullough, 1999; Espinel-Ingroff, 1999). The PCR technique is commonly used for identifying *Candida* species (Antoniadou, 2003). Additionally, several investigators have stated that *C. albicans* can be grouped into several genotypes by pulsed-field gel electrophoresis and random amplification of polymorphic DNA techniques (Pujol, 2002; Lian, 2004). PCR targeting 25S rDNA, which has often been used for genotype analyses of *C. albicans*, allows *C. albicans* to be grouped into genotypes A, B, C, D and E, among which genotype A *C. albicans* constitutes the majority of this yeast (Millar, 2002). However, most studies concerning the genotyping of *C. albicans* have focused on *C. albicans* isolates from infected lesions or normal flora, such as those from the oral cavity, stool and vaginal mucosa. It is expected that a genotyping method with high resolution will be a powerful tool for identifying the dissemination area, infection route or infection source of *C. albicans* for management of candidiasis.

In most cases, cancer patients are infected with opportunistic *C. albicans*, and this fact has led to an increase in the use of antifungal agents, which in turn has resulted in the occurrence of resistance isolates. Antifungal susceptibility is critical in the treatment because *C. albicans* is developing an increasing resistance against antifungal agents (Shokohi, 2011). With no comprehensive documents presented on genotypes and drug resistance of *C. albicans* from Iraq, the present study was designed as a preliminary exploration on genotype dissemination of *C. albicans* isolates from cancer patients and healthy individuals and determine their susceptibility to four different antifungal agents.

2. MATERIALS AND METHODS

Patients: The present study was conducted during the period from January to October 2015. Oral *C. albicans* isolates were collected by oral swabs from the following groups; (i) Fifty cancer patients treated with chemotherapy who exhibited evident oral lesions (70% females and 30% males) constituted the first group. They were being treated as outpatients at the Oncology Unit, Al-Sadder Medical City, Najaf governorate-Iraq, and they were not receiving any

antifungal therapy at the time of sampling. (ii) Twenty five persons from hospitals staff of Al-Sadder Medical City and Al-Zahraa Teaching Hospital (72% males and 28% females) who presented no signs of oral thrush at the time of sampling, referred to as healthy individuals, constituted the second group.

Fungal identification: Samples were cultured on Sabouraud dextrose agar (SDA), (HiMedia, India) at 37°C. Inoculated plates were examined after 24 hours incubation. Isolates from SDA were plated on CHROMagar-Candida (Rambach, France) to ensure detection of light green colony. Cultures were incubated at 37°C for 48 hours. The identity of isolates was confirmed by conventional mycological methods (Freydiere, 2001), such as the germ tube induction test in serum, microscopic morphology, chlamydospore formation in corn meal agar (Oxoid, UK) with tween 80, growth at 45°C and carbon source assimilation by HiCandida identification kit (HiMedia, India) according to the manufacturer's instructions. Each isolate represented a unique isolate from a subject.

Genomic DNA extraction: Yeast cells were cultured on SDA and incubated at 37°C for approximately 48 hours prior to molecular analysis. A single colony of *C. albicans* was suspended in 3 mL of yeast extract-peptone-glucose (YPD) medium for 24 hours at 30°C with agitation. Genomic DNA was extracted using the DNA-Pure Yeast Genomic Kit (BioWorld, USA) according manufacturer's instructions. All DNA samples were stored at -20^{0}C until use.

Genotyping using PCR: The primer pairs used to detect the 25S rDNA were CA-INT-L (5-ATA AGG GAA GTC GGC AAA ATA CCG TAA-3) and CA-INT-R (5-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3) (BioCorp Co., Canada) as described by McCullough. Amplification reactions were performed in 25uL final volume containing 12.5 Robust Hotstart Readymix (Kappa biosystem, South Africa), 20 pmol each of the primers and 5uL DNA template and complete the volume by PCR grade water. The reaction mixtures were subjected to the following thermal cycling parameters in a TECHNE TC-300 (Bibby Scientific, UK): 94ºC for 3 min followed by 30 cycles of 94ºC for 30 sec, 60ºC for 15 sec, 72 º C for 1 min and a final extension at 72ºC for 10 min following the last cycle. All reaction products were characterized by electrophoresis on 1.5% agarose-ethidium bromide gels in 1X TBE buffer at 100V for 60 min. and data analyzed by gel documentation system and gene tool analysis software (SCIE-PLAS, UK). During each run, molecular grade water was included randomly as negative controls and *C. albicans* (ATCC 10231) as reference strain in the study.

Antifungal susceptibility testing: HiComb® MIC tests were performed according to the manufacturer's instructions (HiMedia, India). The antifungal agents used were amphotericin B, fluconazole, itraconazole and ketoconazole. In brief, the inoculum concentration was adjusted to 0.5 in McFarland standard for *C. albicans*. Then, 0.5 mL of this suspension was inoculated onto plates containing Muller-Hinton-Bromothymol blue agar (NCCLS, 2002) using a cotton swab. After a period of 15 min, the HiComb® test strips were applied. The plates incubated at 37°C and read after 24 h. *C. albicans* (ATCC 10231) was used as control strain in this study.

Statistical analysis: The data were entered into SPSS software (Version 13.0, Chicago, USA) and subsequently analyzed, using descriptive statistics, ANOVA and Chi-square for the comparative of data. A p-value of <0.01 was taken as indicative of statistical significance.

3. RESULTS

Only 42 (84.0%) of the 50 oral swabs from cancer patients were found to be *C. albicans* positive, while 13 (52.0%) isolates were recovered from healthy group (Table.1). There were significant differences in *C. albicans* carriage rate in the two groups (p<0.01).

Genotype A was the most common in cancer patients, that represent 33 (78.6%) from total isolates, followed by genotypes B and C (4, 9.5% for each). The remaining one (2.4%) isolate was categorized as genotype T, the study considered this isolate to belong to a new genotype (Table.2; Figure.1). Genotype A *C. albicans* constituted the completely isolates in healthy group (Figure.2).

The antimicrobial potency of selected antifungal against in the isolates are summarized in table.3. The resistance effects of isolates to antifungal agents are different. The resistance to fluconazole and ketoconazole among all isolates was 18.2%. Additionally, 14.6% of isolates display itraconazole resistance. The most effective antifungal was amphotericin B (3.6% resistance). Of the genotype A *C. albicans* isolates obtained from cancer patients, 6 (18.2%) were resistant to ketoconazole, 5 (15.2%) were resistant to fluconazole and itraconazole as well as 1 (3.0%) was resistant to amphotericin B. While, 1 (25.0%) isolate of genotypes B and C *C. albicans* exhibited cross resistant to fluconazole and ketoconazole, respectively. The genotype T isolate was susceptible to all antifungal-tested. The susceptibility patterns of the genotype A isolates obtained from healthy individuals exhibited cross resistant to fluconazole, itraconazole (23.1%) and ketoconazole (15.4%) as shown in table.3.

Table.1. Frequency of *C. albicans* isolated from oral swabs of cancer and healthy groups

Individuals	No. of samples	No. of isolates (%)	Males (%)	Females (%)
Cancer patients	50	42 (84.0)*	14 (33.3)	28 (66.7)
Healthy persons	25	13 (52.0)	8 (61.5)	5 (38.5)
Total	75	55 (73.3)	22 (40.0)	33 (60.0)

*p<0.01

Table.2. The distribution of genotypes *C. albicans* among study groups

Individuals	Genotype A (%)	Genotype B (%)	Genotype C (%)	Genotype T (%)
Cancer patients	33 (78.57)	4 (9.52)	4 (9.52)	1 (2.38)
Healthy persons	13 (100.0)	0 (0)	0 (0)	0 (0)
Total	46 (83.64)*	4 (7.27)	4 (7.27)	1 (1.82)

*p<0.01

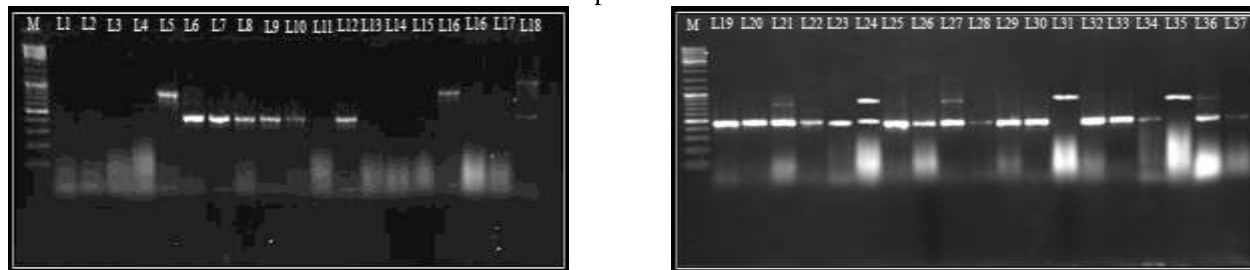

Figure.1. Ethidium bromide-stained agarose gel of PCR amplified products of *C. albicans* genotypes for cancer patients

Lane (M), DNA molecular size marker (100 bp ladder); lanes (L6-L10, L12, L19, L20, L22, L23, L25-L26, L28-L30, L32-L34, L37) show genotype A with (450bp) PCR product size; lanes (L5, L16, L31, L35) show genotype B with (850 bp) PCR product size; lanes (L21, L24, L27) show genotype C with (450 bp, 850 bp) PCR product size; lane (L18) show genotype T with (450 bp, 1067 bp) PCR product size. (1.5% agarose gel, 100V-1hour).

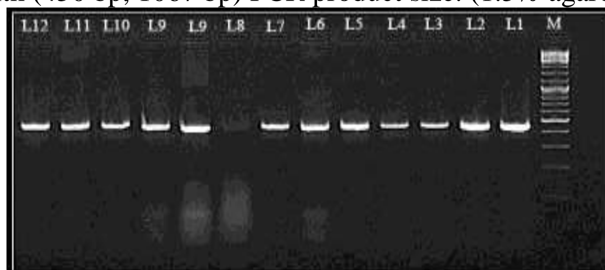

Figure.2. Ethidium bromide-stained agarose gel of PCR amplified products of *C. albicans* genotypes for healthy group

Lane (M), DNA molecular size marker (100 bp ladder); all lanes show genotype A with (450 bp) PCR product size. (agarose gel 1.5%, 100V-1hour).

Table.3. Antifungals resistance of *C. albicans* isolated from mouth swabs of cancer and healthy groups

Group	Genotype	Flu (%)	Kt (%)	It (%)	Amp-B (%)
Cancer Patients	A (n=33)	5 (15.15)	6 (18.18)*	5 (15.15)	1 (3.03)
	B (n=4)	1 (25.0)	1 (25.0)	0 (0)	0 (0)
	C (n=4)	1 (25.0)	1 (25.0)	0 (0)	1 (25.0)
	T (n=1)	0 (0)	0 (0)	0 (0)	0 (0)
	Total (n=42)	7 (16.66)	8 (19.04)*	5 (11.9)	2 (4.76)
Healthy Persons	A (n=13)	3 (23.07)	2 (15.38)	3 (23.07)	0 (0)

*p<0.01, Flu=fluconazole, Kt=ketoconazole, It=itraconazole, Amp-B =amphotericin-B

DISCUSSION

Cancer patients are highly susceptible to hospital acquired *C. albicans* infections. This microorganism is increasingly implicated in serious immuno-compromised infections. There are few detail epidemiological and antifungal studies in Iraq about this subject, since our study focused on detection of *C. albicans* isolates rather than other *Candida* species. The results revealed that the isolation rate of *C. albicans* in cancer patients were 84.0%. Our study revealed that the *C. albicans* isolates were frequented in mouth swabs of cancer patients. In one report, *C. albicans* and non *C. albicans* infections account for most 15% to 70% of the infection accruing in cancer patients (Krcmery and Barnes, 2002). Goncalves (2006), has shown great variability in the number of patients whose are

colonized by *C. albicans*. This may be due, at least in part, to the sampling method and to the fact that *C. albicans* isolated in the present study did not necessarily correlate with clinical evidence of clinical *Candida* infections.

Candida albicans is a normal commensal of the mouth and generally causes no problems in healthy people, it is frequently isolated from the human mouth, yet few carriers develop clinical signs of candidiasis (Prescott, 2008). However, in this study 52.0% of isolates were obtained from mouth swabs of apparently normal persons from hospitals staff. The high isolation rate in medical staff may be due to the ability of isolates to survive outside a living host and remain in the environment for long periods of time, thus being able to contaminate skin surfaces and the hands of healthcare professionals. As well, Hota (2004) also reported this observation. This may be explaining this percentage of *C. albicans* isolated from normal person.

Molecular typing of an infectious agent is important for epidemiological studies and for the development of appropriate infection control strategies. Because of the characteristics of *C. albicans* and the need for better understanding its epidemiology, molecular techniques are employed to provide the characterization of the isolates. Such a characterization can be used to track the organism within a host, between hosts, or between host and inanimate objects, or to associate particular strains with various anatomic sites, particular disease entities, or particular host features. In Iraq, all clinical laboratories are not fully aware of the importance of molecular typing *C. albicans* and how to detect them; laboratories may also lack the resources to epidemiological study and curb the spread of these types. Methods for detecting genotyping of *C. albicans* are technically demanding for Iraq clinical laboratories. However, genotypes that cause disease often go undetected and have been responsible for several nosocomial infections. To our knowledge, until now no published researcher has described the distribution of genotypes within *C. albicans* isolates in Iraqi hospitals. However different types have been identified in this study, but not phenotypic test can differentiate among them, a fact which creates problems for surveillance and epidemiological studies.

In this report the genotyping of *C. albicans* isolates collected from cancer patients and health individuals, showed that the majority of the isolates have identical genotypes, with a single genotype A predominating in most isolates. While, genotypes B and C were less frequented in cancer patients group and absent in healthy group. In a hospital setting when isolates derived from different patients are genetically identical, it may be generally assumed that cross-infection or colonization has occurred or that the patients were infected or colonized by exposure to a common source. In this study, genotyping A *C. albicans* isolates recovered from different patients and healthy persons who were in the same hospitals suggests may be a clonal spread of one isolate, belonging to genotype A that caused colonization or infection of these individuals and even though horizontal transmission is the most probable explanation for this cluster of cases, environmental, hand and pharynx cultures of hospitals healthcare workers. At the same time, they could be assumed as a probable hypothesis to understand the source of infection or colonization and the route of transmission. Several published studies have reported a predominance of genotype A in clinical isolates of *C. albicans* (Bii, 2009; Gurbuz and Kaleli, 2010). However, the rate of genotype A that detected in this study was located within the range from 50 to 90% that reported in previous studies (Iwata, 2006; She, 2008).

Present study described a new genotype symbolized as T in one isolate, established on the basis of the presence of differences in PCR amplified products. This isolate was obtained from cancer patient. However, no data was available in previous reports about this pattern of genotype. In recent study, Bii (2009), detected a new genotype pattern in Kenya (genotype AB) based on 25s rDNA amplification products, but these amplified products was differ from product size detected in the present study. This pattern conceder as transient genotype or it may be intermediate form occurring during the transition from genotype to other genotype or as a result of the sexual reproduction (sexual mating) between genotypes, however these opinion strongly supported by McCullough (1999), and Gurbuz and Kaleli (2010). Further researches are required to indicate if this genotype a stable genetic form or whether its stage of genetic transient form.

Although amphotericin B is widely used in clinical practice in Iraq, present findings showed that *C. albicans* isolates were highly sensitive to amphotericin B. No data was available in relation to resistance to amphotericin B in Iraqi hospitals. In recent study, Nassir (2010), found that 2.6% of *C. albicans* isolates were resistant to this agent. Amphotericin B complex with ergosterol in the membranes causes a cascade of cell disturbing events. Amphotericin B resistant *Candida* isolates have a marked decrease in their ergosterol content (Arikan, 2002). The study suggested that intensive use of this drug in hospitals may be lead to changing in ergosterol structures and this may explain the current rate of resistance in this study.

In our country, little consideration has been paid to azole resistance isolates (Nassir, 2010; Al-Malaky, 2015). Overall resistance of all *C. albicans* isolates to fluconazole was 18.2%, most investigations have focused on tests with fluconazole against pathogenic yeasts, particularly since reports have appeared suggesting that *Candida* isolates with reduced fluconazole susceptibilities are being encountered clinically after fluconazole treatment (Badiee, 2009; Rizvi, 2011). Decreasing susceptibility to the fluconazole in hospitals may be due to the increasing incidence of colonization and infection with *C. albicans*. The reason for the high fluconazole resistance may be also explained by the fact that fluconazole was prescribed to the most immune compromised patients as a standard care in our country.

Consequently, *in vitro* testing of the susceptibility of *C. albicans* to antifungal agents will likely play an ever-increasing role in the appropriate selection of antifungal agents for the treatment of fungal infections. Several reports describing *in vitro* and clinical resistance to fluconazole developing during antifungal therapy have been published elsewhere (Sanguineti, 1993; Vazquez, 1993).

In the present study 18.2% of all *C. albicans* isolates were determined resistant to ketoconazole, a finding which higher than some other studies results (Badiee, 2009; Satana, 2010). The mode of action of ketoconazole is similar to fluconazole (Muller, 2000). Because of similarity in mode of action and gene regulation with fluconazole, present study supposed that the resistant cases duo to cross resistant with other azoles.

Our study showed a high itraconazole resistance (14.6%) among *C. albicans* isolates, the reasons for resistance might include incomplete therapy, overgrowth of resistant isolates, and induction of drug resistance in the particular species, colonization and subsequent infection with a resistant organism. Other investigators (Powderly, 1994; Rex, 1995) also report these statements.

With no comprehensive data available on antifungal resistance and genotypes of *C. albicans* from Iraq, the present study showed a clear predominance of the genotype A in both cancer patients and healthy individuals groups. In addition, there was no specific association between the genotypes of *C. albicans* with antifungal resistance. For example, 18.2%, 25.0% and 25.0% of the genotype A, genotype B and genotype C *Candida albicans* isolates from cancer patients were resistant to ketoconazole, respectively. Genotype A isolates are known to lack the group I intron in the 25S rRNA gene (rDNA), whereas genotype C isolates possess the intron and this difference in occurrence of the intron may suggest that it does not play a role in azole and polyenes resistance (Mercure, 1993). Resistant to azole and polyenes are related to ERG11 gene (Ge, 2010), *CdCDR* and *CdMDR1* genes (Pinjon, 2004) and location of these genes were not related to 25S rDNA loci used in *C. albicans* genotyping in this study, so, this method is not useful to study the resistant cases of *C. albicans* to antifungal agents. This however, has to be investigated further.

4. CONCLUSION

As a final point, the use of molecular diagnostic tests to detect of *C. albicans* genotypes, is expected to provide more accurate means by which to understand the epidemiology of isolates infections. Therefore, our study believes that description of active pathogens in *Candida* infections at the genotype level and research on antifungal sensitivity will be very useful in epidemiology, managing treatment, and preventing resistance development in hospitals.

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