Genotyping and Antifungal Vulnerability of Candida albicans Isolated from Cancer Patients in Dewaniyeh Governorate.

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Abstract
Background: Candida infections are one of the major causes of morbidity in cancer patients.
Aim: To determine the genotype and antifungal susceptibility of C. albicans isolated from cancer patients and compared with that from healthy individuals.
Materials and methods: Oral swabs collected from cancer patients and healthy subjects were screened for the occurrence of C. albicans. Isolates were identified to species level by the conventional mycological methods. Genotypes were determined with use of 25S rDNA PCR analysis. Susceptibility testing was performed using HiCombo MIC technique.
Results: Phenotypic examination showed that oral C. albicans was detected in 90% of cancer patients and 50% of healthy controls. PCR shows that the isolates of A, B, C and T genotypes, among which genotype A C. albicans was the predominant genotype. Genotype A C. albicans recognized the entirely isolates in healthy group. Isolates were most sensitive to amphotericin B. Occurrence of resistance to amphotericin B was the lowest followed by itraconazole. However, the isolates demonstrated a high rate of resistance to ketoconazole and fluconazole.
Conclusion: Based on these results, 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.
Key words: Candida albicans; PCR; Cancer, Itaraconazole, Amphotericin B; Ketokonazole; 25S rDNA; Antifungal agents.

Introduction
Although 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 [1,2]. C. albicans is the most common cause of oral candidosis in cancer patients receiving radiotherapy and/or chemotherapy [3].

Fast recognition of the etiological agent is important for the success of treatment so that molecular techniques are increasingly used to shorten identification time and accuracy [4]. Molecular typing of an infectious agent is important for the development of suitable infection control strategies [1]. To understand the epidemiology of C. albicans infection, various molecular techniques have been used for strain description [1,5]. The PCR technique is commonly used for identifying Candida species [6]. 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 [7,8]. 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 [9]. 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 candidosis.

The majority of cancer patients are infected with opportunistic C. albicans, and this 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 [10]. 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 from Dewanyeh and to determine the susceptibility of the isolates to four different antifungal agents.

Materials and Methods
Study Population
The present study was conducted in the Department of Microbiology, Faculty of Medicine, University of Al-Qadisiyah, during the period from January to October 2015. Oral C. albicans isolates were collected by oral swabs from the following groups; (i) Hundred cancer patients treated with chemotherapy who exhibited evident oral lesions (70% females and 30% males) constituted the first group. These patients were being treated as outpatients at the Oncology Unit in Al-Dewanyeh teaching hospital, Dewanyeh Governorate-Iraq, were not receiving any antifungal therapy at the time of sampling. (ii) Fifty persons from hospitals staff of Al-Dewanyeh teaching hospital (70% males and 30% females) who presented no signs of oral thrush at the time of sampling, referred to as healthy controls, constituted the second group.

Candida isolation and species 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 [11], 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°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 (McCullough et al., 1999). Amplification reactions were performed in 25μl final volume containing 12.5 Robust HotstartReadymix (Kappa biosystem South Africa), 20 pmol each of the primers and 5μL 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): 94°C for 3 min followed by 30 cycles of 94°C for 30 sec, 60°C for 15 sec, 72°C for 1 min and a final extension at 72°C for 10 min following the last cycle. All reaction products were characterized by electrophoresis on 1.5% agarose-ethidium bromide gels in 1× 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 [12] using a cotton swab. After a period of 15 min, the HiComb® test strips were applied. The plates were 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.

Results

90 (90%) of the 100 oral swabs from cancer patients were found to C. albicans positive, while 25 (50%) 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 70 (77.7%) from total isolates, followed by genotypes B (9 isolates) and C (9 isolates), (10% for each). The remaining two isolates (3.3%) was categorized as genotype T, the study considered this isolate to belong to a new genotype (Table 2). Genotype A C. albicans constituted the completely isolates in healthy group.

The antimicrobial activity of selected antifungal agents in the isolates is summarized in Table (3). The resistance of the isolates to antifungal agents is different. The resistance to fluconazole, ketoconazole and itraconazole among all isolates was 16.8%. The most effective antifungal was amphotericin B, where resistance, reported in (2) patients (3.03% resistance).

Of the genotype A C. albicans isolates obtained from cancer patients, 10 (14.2%) were resistant to fluconazole and itraconazole, 12 (17.1%) were resistant to
ketoconazole as well as 2 (1.4%) were resistant to amphotericin B. While, 2 (22.0%) isolate of genotypes B and C of \textit{C. albicans} exhibited cross resistant to fluconazole and ketoconazole, respectively. The genotype T isolate was susceptible to all antifungal-tested. The resistance patterns of the genotype A isolates obtained from healthy individuals, exhibited resistant to fluconazole, itraconazole [6 persons for each of them (24%)], while 4 (16%) exhibited ketoconazole resistance as shown in Table (3).

**Discussion**

Cancer patients are highly susceptible to hospital acquired \textit{C. albicans} infections. This microorganism is increasingly implicated in serious immunocompromised infections. There are few detail epidemiological and antifungal studies in Iraq about this subject, since our study focused on detection of \textit{C. albicans} isolates rather than other Candida species. The results revealed that the isolation rate of \textit{C. albicans} in cancer patients were 90%. Our study revealed that the \textit{C. albicans} isolates were frequented in mouth swabs of cancer patients. In one report, \textit{C. albicans} and non \textit{C. albicans} infections account for most 15% to 70% of the infection accruing in cancer patients [13,14] has shown great variability in the number of patients whose are colonized by \textit{C. albicans}. This may be due, at least in part, to the sampling method and to the fact that \textit{C. albicans} isolated in the present study did not necessarily correlate with clinical evidence of clinical Candida infections.

\textit{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 candidosis [15]. 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 [16] reported this observation. This may be explaining this percentage of \textit{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 \textit{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 \textit{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 \textit{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 \textit{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* [17,18]. 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 [19,20].

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. [17] 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 concedes 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 this opinion strongly supported by others findings [18,21].

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 our hospital. In a study in Iraq, Nassir [22] 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 [23]. 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 [22]. 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 [24,25]. 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 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 [26,27].

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 [24,28]. The mode of action of ketoconazole is similar to fluconazole [29]. Because of similarity in mode of action and gene regulation with fluconazole, present study supposed that the resistant cases are due 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 [30, 31] also report same results.

With no comprehensive data available on antifungal resistance and genotypes of C. albicans from Iraq, the present study was designed as a preliminary investigation on genotype distribution of C. albicans isolates from cancer patients and healthy individuals in Najaf and determines the susceptibility of the isolates to four different antifungal agents. Our study showed a clear predominance of the genotype A in both 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 of C. 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 [32]. Resistant to azole and polyenes are related to ERG11 gene [33], CACDR and CdMDR1 genes [34] 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.

At the end, epidemiological studies of the isolate of C. albicans infection requiring the use of molecular diagnostic test to detect C. albicans genotypes. Additionally, it is known that C. albicans increase in cancer patients and that this species has a high resistance to antifungal drugs [35].

In conclusion, genotype level detection of active pathogens in Candida infections and research on antifungal susceptibility will be very useful in managing treatment, decreasing the development of resistance and it can be very useful subsequent epidemiological studies.

References:


21. McCullough, M.J., Clemons, K.V. & Stevens, D.A. Molecular and phenotypic characterization of genotypic Candida albicans subgroups and comparison with

22. Nassir, N.I. Incidence of Candida albicans isolates from oral and vaginal candidosis, study of their susceptibility and cross-resistance to some antifungal agents. MSc Thesis, College of Medicine, Al-Qadisiya University, 2009.


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

<table>
<thead>
<tr>
<th>Individuals</th>
<th>No. of samples</th>
<th>No. of isolates (%)</th>
<th>From males (%)</th>
<th>From females (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patients</td>
<td>100</td>
<td>90 (90.0)*</td>
<td>30 (33.3)</td>
<td>60 (66.6)</td>
</tr>
<tr>
<td>Healthy persons</td>
<td>50</td>
<td>25 (50.0)</td>
<td>15 (60.0)</td>
<td>10 (40.0)</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>115 (76.6)</td>
<td>45 (39.1)</td>
<td>70 (60.8)</td>
</tr>
</tbody>
</table>

*p < 0.01

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

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Genotype A (%)</th>
<th>Genotype B(%)</th>
<th>Genotype C(%)</th>
<th>Genotype T(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patients</td>
<td>70 (77.7)</td>
<td>9 (10.0)</td>
<td>9 (10.0)</td>
<td>2 (3.3)</td>
</tr>
<tr>
<td>Healthy persons</td>
<td>25 (100.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>95 (83.64)*</td>
<td>9 (7.82)</td>
<td>9 (7.82)</td>
<td>2 (1.73)</td>
</tr>
</tbody>
</table>

*p < 0.01

Table 3: Antifungal resistance of C. albicans isolated from mouth swabs of cancer and healthy groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Genotype</th>
<th>Flu(%)</th>
<th>Kt(%)</th>
<th>It(%)</th>
<th>Amp-B(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer Patients</td>
<td>A (n=70)</td>
<td>10 (14.28)</td>
<td>12 (17.14)*</td>
<td>10 (14.28)</td>
<td>2 (2.57)</td>
</tr>
<tr>
<td></td>
<td>B (n=4)</td>
<td>1 (25.0)</td>
<td>1 (25.0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B (n=9)</td>
<td>2 (22.2)</td>
<td>2 (22.2)</td>
<td>0</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td></td>
<td>T(n=2)</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total (n=90)</td>
<td>14 (15.55)</td>
<td>16 (17.77)*</td>
<td>10 (11.11)</td>
<td>4 (4.44)</td>
</tr>
<tr>
<td>Healthy Persons</td>
<td>A(n=25)</td>
<td>6(24.0)</td>
<td>4(16.0)</td>
<td>6(24.0)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

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