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ORIGINAL ARTICLE

Molecular Identification and In-Vitro Susceptibility of *Candida albicans* and *C. dubliniensis* Isolated from Immunocompromised Patients

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Abstract

Background: Yeasts are increasingly implicated in serious systemic infections. The aim of this study was to identify *Candida albicans* and *C. dubliniensis* from isolates of immunocompromised patients and evaluate the in vitro antifungal activities of them against antifungal agents.

Methods: One hundred and seventy eight *C. albicans* were isolated by routine methods from 403 immunocompromised patients. All isolated *C. albicans* were inoculated on CHROMagar *Candida* medium. The carbohydrate assimilation patterns of all the isolates were studied, using the API 32C system. To identify *C. albicans* and *C. dubliniensis,* PCR was done by specific primers. The susceptibility test for the isolates was performed by a broth microdilution assay, according to the Clinical and Laboratory Standard Institute.

Results: Of 178 isolates *C. albicans*, six were *C. dubliniensis* with PCR assay, and 7% were resistant to amphotericin B, 4.6% to fluconazole, 7% to itraconazole, 1% to nystatin, 2.3% to voriconazole, and 7% to ketoconazole. None of the *C. dubliniensis* isolates were resistant to the six anti-fungal agents.

Conclusion: It would be convenient to carry out antifungal susceptibility studies in order to establish the in-vitro activities of antifungal agents against local isolates and also to detect shifts toward resistance as early as possible.

Keywords: *Candida*; Fluconazole; Amphotericin B; Ketoconazole; Nystatin; Antifungal susceptibility; Immunocompromised

Introduction

Resistance among *Candida spp.* to antifungal drugs is an increasing problem in immunocompromised patients. Although there has been a significant increase in the number of reported infections caused by yeasts of the genus *Candida, C. albicans* is the most frequent isolated *Candida* species. However, in recent years, the number of infections due to non-albicans *Candida* species has increased significantly.

C. dubliniensis was first described as a distinct taxon in 1995.[1] It is chlamydospore-positive, germ tube-positive species, having many characteristics in common with *C. albicans*. Although *C. albicans* and *C. dubliniensis* are phenotypically very similar, they differ in their carbohydrate assimilation profiles, growth patterns at elevated temperatures, and intracellular *B*-glycosidase activities.[2,3]

In view of the recurrent nature of fungal infections in immunocompromised patients, treated by immunosuppressive drugs or chemotherapy, many sufferers receive repeated courses of antifungal medications. Prolonged or repeated exposure to antifungal drugs may be associated with the emergence of antifungal resistance among strains of *C. Albicans.*[4] Inherent decreased susceptibility to commonly used antifungal drug therapy may be an important factor in the emergence of some *Candida* species as opportunistic pathogens.

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The aim of this study was to identify *Candida* species, previously recognized as *C. albicans*, by phenotypic and molecular methods, and to evaluate the in vitro activities of fluconazole, amphotericin B, ketoconazole, itraconazole, voriconazole, and nystatin against isolates.

Materials and Methods

From October 2003 to March 2007, 178 *C. albicans* were isolated by the routine methods (Germ tube and chlamydospore production) from 403 immunocompromised patients in Ttransplant and Oncology wards in Nemazee Hospital affiliated to Shiraz University of Medical Sciences, southern Iran. At the outset, we checked colonization of *Candida* (by providing swabs from the mouth, and vaginal and rectal tissues). Some clinical evidence of fungal infections was detected and finally *C. albicans* were isolated from clinical samples (Table 1). *Candida albicans* were isolated from patients consisting of 90 males and 88 females. All the patients were HIV-negative. The samples were stored at -70°C in 10% glycerol, for different periods of time.

All isolated *C. albicans* (178) were cultured on potato dextrose agar (OXOID LTD, Basingstoke, Hampshire, England) twice for 24h-48h at 35°C to ensure viability and purity. The isolates were then inoculated on CHROMagar *Candida* medium (CHROM agar microbiology, Paris, France) and incubated at 30°C for 4-7 days with daily examination looking for light green colonies (for *C. albicans*) and dark green colonies (for *C. dubliniensis*).

Light and/or dark green colonies were incubated in fresh human sera for 3h at 37°C and evaluated for germ tube forming. In order to investigate chlamydospore production, all *C. albicans* isolates were cultured on corn meal agar (Difco^{TM}) tween 80 for 7 days. The carbohydrate assimilation patterns of all the isolates were studied, using the API 32C system according to the manufacturer's procedure (BioMe'rieux, France).

Susceptibility of *C. albicans* isolates to fluconazole, amphotericin B, ketoconazole, nystatin (SIGMA-Aldrichemie GmbH-Steinheim, Germany), itraconazole (Janssen Pharmaceutical, Beerse, Belgium), and voriconazole (Pfizer, United Kingdom) was determined by a broth microdilution assay according to the Clinical and Laboratory Standard Institute (CLSI; formerly NCCLS).[5] Stock solutions of the drugs were prepared in di-methyl sulfoxide (Merck, Germany) or water.

Two CLSI quality control strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were tested each time and a set of clinical isolates were evaluated.[6] Final concentrations of amphotericin B ranged from 16 to 0.032 $\mu g\ ml^{-1}$, fluconazole from 64 to 0.125 $\mu g\ ml^{-1}$, nystatin from 18.5 to 0.035 and of itraconazole, ketoconazole and voriconazole from 16 to 0.032 $\mu g\ ml^{-1}$. In each serial, one control positive with no drugs and one negative control with no fungal suspensions were designed. The plates were sealed and incubated at 35°C after 24h and 48h of incubation, visual MIC end-points were determined with the aid of a magnifying mirror. Visual end-points were determiners as described in the CLSI M 27-A2 that recommended end-point for azoles as the lowest drug concentration with a prominent decrease in turbidity (inhibitory concentration that gives 50% growth reduction), while for amphotericin B and nystatin, the MICs was the drug concentration showing a complete inhibition of growth.

The amount of growth in each tube was compared to that of the growth of positive control. Antifungal activity was expressed as the MICs of each isolate to the drug. The following resistance breakpoints were used according to CLSI guidelines[5] or based on previous investigations.[7-10]

Genotypic Identification Tests

The size of fragment amplified by primers CAL5 and NL4CAL for *C. albicans*, CDU2 and NL4CAL for *C. dubliniensis* was 175 bp which corresponds to

Table 1: Sites of body for the isolated candida species

Sites of *Candida* isolated	No. (%) *(C. albicans)*	No. (%) *(C. dubliniensis)*	Total
Mouth	138 (77.5)	2 (1.1)	140 (78.6)
Vagina	8 (4.5)	0 (0.0)	8 (4.5)
Sputum	18 (10)	4 (2.3)	22 (12.3)
Blood	4 (2.3)	0 (0.0)	4 (2.3)
Abdominal	4 (2.3)	0 (0.0)	4 (2.3)
Total	172 (96.6)	6 (3.4)	178 (100)

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those observed by Mannarelli *et al.*; thus, allowing for the identification of the two respective species *C. albicans* and *C. dubliniensis*.[11] Pure culture was prepared by re-suspending a loopful of organism in 200 µl of sterile water, and DNA was extracted as previously described.[12]

Two pairs of the primers were used:[11] one for *C. dubliniensis* sense: CDU2, 5′-AGT TAC TCT TTC GGG GGT GGC CT- 3′; and anti-sense NL4CAL 5′-AAG ATC ATT ATG CCA ACA TCC TAG GTA AA-3′ and another for *C. albicans*, sense, CAL5 5′-TGT TGC TCT CTC GGG GGC GGC CG- 3′; and anti-sense NL4CAL, 5′- AAG ATC ATT ATG CCA ACA TCC TAG GTA AA-3′ (Figure 1). Amplification and electrophoresis were done as done by Mannarelli *et al.*[11] All the primers and probes were synthesized by TIB MOLBIOL (Berlin, Germany). The same conditions were used with *C. dubliniensis* primers in the isolates suspicious to *C. albicans*. Differentiation between *C. albicans* and *C. dubliniensis* was achieved based on both phenotypic characteristics and the PCR test.

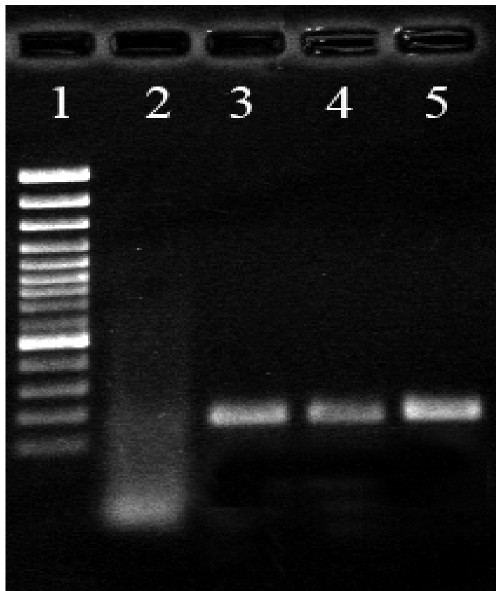

Fig. 1: *Candida dubliniensis* DNA were amplified with specific primers, and amplicons were detected by gel electrophoresis and ethidium bromide staining
1: 100bp DNA size marker, 2: Negative Control, 3: positive control, 4 and 5: *C. dubliniensis*

The data were entered into SPSS software (version 11.5, Chicago, IL, USA) and subsequently analyzed, using descriptive statistics and cross tabulation.

Results

All 178 *C. albicans* strains isolated were able to produce germ tube and produced chlamydospore on cornmeal agar tween 80. Six isolates were considered as possible *C. dubliniensis* by assimilation patterns (pattern API 32 C: 7143.1400.15).

Of 178 isolates diagnosed previously as *C. albicans*, six were *C. dubliniensis* with polymerase chain reaction test. There was no significant difference between reading plate in 24h and 48h of incubation (94% the same); thus we reported reading plates on 48h. Of 172 *C. albicans* isolates, 93% were sensitive and 7% were resistant to amphotericin B, 93% were sensitive, 2.3% susceptible-dose dependent, 4.6% were resistant to fluconazole, 71% were sensitive, 22% susceptible-dose dependent, and 7% were resistant to itraconazole, 99% were sensitive, and 1% were resistant to nystatin, 95.4% were sensitive, 2.3% susceptible-dose dependent, and 2.3% were resistant to voriconazole , 93% were sensitive, 7% were resistant to ketoconazole. The in vitro susceptibilities of *C. albicans* isolates to antifungal agents are summarized in Table 1 and 2.

All the *C. dubliniensis* strains were sensitive to amphotericin B, fluconazole, nystatin, voriconazole, ketoconazole, and susceptible- dose dependent to itraconazole.

Discussion

As noted previously,[13,14] the data demonstrated the sustained importance of *C. albicans* as an etiologic agent of fungal infections in normally sterile sites. The frequency of *C. albicans* as a cause of infection may vary in different institutions.

C. dubliniensis isolates have been primarily recovered from oral and mucosal surfaces especially in HIV-positive patients. However, there have been a number of recent reports of its isolation from non-HIV-positive patients.[1,15] In-depth epidemiological studies are required to determine the precise clinical importance of this organism, particularly because of its ability to develop in vitro resistance to fluconazole.[16]

Recognition of phenotypic characteristics that allow simple detection and differentiation of *C. dubliniensis* remains a problem in routine yeast identification. Growth at 45°C alone and use of CHROM agar *Candida* by itself was not sufficiently trustworthy.[17] Colonies of *C. dubliniensis* often have an

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Table 2: Repartitions of the 178 yeast isolates for amphotricin B, fluconazole, itraconazole, voriconazole, ketoconazole and nystatin

	C. albicans			*C. dubliniensis*		
	S	**SDD**	**R**	**S**	**SDD**	**R**
AmphotericinB	160	0	12	6	0	0
Fluconazole	160	4	8	6	0	0
Itraconazole	122	38	12	3	3	0
Voriconazole	164	4	4	6	0	0
Ketoconazole	160	0	12	6	0	0
Nystatin	170	0	2	6	0	0

S: sensitive, SDD: susceptible dose dependent, R: resistant; Resistance is defined as the following MICs (in micrograms per milliliter): Amphotericin B>1; Fluconazole ≥64; Itraconazole ≥1; Voriconazole ≥8; Ketoconazole ≥4; Nystatin ≥16.

unusually dark green color within 48 h at 37°C when freshly isolated from clinical materials on the differential medium CHROM agar *Candida*, but this property is not retained in subculture or when incubation is prolonged for 4 days or more.[17]

The variation of the substrate reactivity profiles obtained by using the commercial identification kit (API ID 32C system) showed an atypical *C. albicans* profile and was regarded as possible isolates of characteristics found by different methods used. Gales and *et al.* reported 23 of 100 *C. albicans* isolates (23%) exhibiting poor or no growth at 45°C. Xylose (XYL) and α-Methyl-D-Glucoside (MDG) tests contained within the API 20C AUX system were both negative for all 66 *C. dubliniensis* isolates and positive for 98 (XYL) and 56 (MDG) of the 100 *C. albicans* isolates.[18] Therefore, sufficient and reasonable discrimination of molecular diagnosis between both species was feasible.

Mirhendi *et al.* reported 140 clinical isolates from Medical Mycology Department, Tehran University of Medical Sciences, Tehran, Iran, identified as *C. albicans* by CHROM agar *Candida*, none of which was *C. dubliniensis* by RFLP method.[19]

Coleman *et al.* reported that approximately 2% of the isolates from asymptomatic healthy individuals and approximately 17% of those from HIV-infected individuals which had originally been identified as *C. albians* were in fact *C. dubliniensis.*[2] Toraman *et al.* and Colombo *et al.* reported that 9.1% and 2% of germ tub-positive *Candida* strains were identified as *C. dubliniensis* by molecular identification.[20,21] In Brazilian yeast stock collection from 1994 to 2000, 11 isolates were identified as *C. dubliniensis* and all of them were susceptible to azoles and amphotericin B and 17 (3%) *C. albicans* isolates were susceptible dose-dependent or resistant to azoles.[22] Most isolates of *C. dubliniesis* were susceptible in-vitro to commonly used antifungal agents; however, resistance to fluconazole has been reported.[23] Among *C. dubliniensis* isolated in Venezuela, 19% showed MICs above 64 µg/ml to fluconazole.[24]

In the present study, 6 out of 178 samples (3.4%), previously identified as *C. albicans,* were actually *C. dubliniensis* being sensitive to all selected antifungal agents (for itraconazole some isolates were susceptible and dose dependent) (Table 2). Comparison of in-vitro antifungal susceptibility patterns of *C. albicans* isolated in different studies is shown in Table 3.

Fluconazole is a triazole agent that is established as a first line antifungal agent for the treatment of candidiasis. A study from South Africa reported 100% susceptibility to fluconazole among 466 isolates of *C. albicans.*[8] Of all 73 *C. albicans* isolates collected over a 5-year period (2001-2005), only two isolates (one from 2003 and one from 2004) showed resistance to fluconazole.[25] Matar *et al.* (2003) showed that more than 96% of the isolates were susceptible to fluconazole by the reference method.[26] In the present study, 4.6% of the isolates were resistant to fluconazole and in the range of 0.125-64 µg/ml MIC_{90} for *C. albicans* against fluconazole was 0.25 µg/ml. The results are similar to those reported by a few investigators[8,26,27] and different from those of some others.[28-30]

Voriconazole is a new triazole drug with very few strains resistant to it. Upon evaluating the susceptibility in previous studies, it was found that in 1997; 98%, in 1998; 99% and in 1999; 100% of *C. albicans* were susceptible to voriconazole.[31] None of the isolates in Huiet *et al.*'s study showed resistance to voriconazole.[25] In the present study, 2.3% of the isolates

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Table 3: Comparison between in-vitro antifungal susceptibility patterns of *C. albicans* (based on references)

Antifungal	MIC(ug/ml)			Reference
	Range	50%	90%	
AmphotericinB	0.032-1	0.12	0.5	Present study
		1	1	8
		0.5	1	31
Fluconazole	0.125-64	0.12	0.25	Present study
		0.25	0.25	8
		0.125	0.25	27
		--	1.0	28
		1.0	4.4	29
		--	0.5	30
		0.12	2	31
Itraconazole	0.032-1	0.125	0.5	Present study
		0.03	0.6	8
		0.03	0.5	31
Voriconazole	0.032-4	0.032	0.125	Present study
		0.015	0.12	8
		0.031	0.031	27
		--	0.015	30
		0.015	0.12	31
Ketoconazole	0.032-4	0.032	0.125	Present study
		0.007	0.03	8
		0.03	0.5	31
Nystatin	0.035-18.5	2.3	4.6	Present study
		4	8	8
		1	1	31

were resistant to voriconazole, which is very expensive and very limited number of patients can afford it in Iran. This might be why there is the lowest resistance against this drug among immumocompromised patients. Itraconazole and voriconazole showed very close in-vitro activities against the tested yeast isolate. In spite of this, there were some differences in susceptibility among the isolates within the same fungal species. Lopez *et al.* reported that MICs of 0.125 µg/ml was observed for itraconazole and voriconazole against yeasts and molds fungus.[32] Bagg *et al.* (2003) in 93 *C. albicans* isolated from patients with advanced cancer reported resistance of about 3% to fluconazole and 4% to itraconazole.[33] In the present study, 7% of the isolates were resistant to itraconazole and MIC_{50} and MIC_{90} for the isolates were 0.125 and 0.5 ug/ml. Once immunocompromised patients with fungal infections or suspicious to fungal infections were discharged from hospital, they received itraconazole for a long time which may cause high resistance to itraconazole in the *C. albicans* isolated from them.

Amphotericin B is traditionally used in topical forms, although it may be administrated systemically for the treatment of systemic infections in the hospitalized patients. Citak *et al.*, found that amphotericin B was an effective anti-fungal agent that could be used against all *Candida* isolates.[34] But in Blignaut *et al.*, (2002) 8.4% of *C. albicans* isolated were resistant to amphotericin B.[8] In the present study, 7% of the isolates were resistant to amphotericin B, and there was no strain that exhibited MICs greater than 4 µg/ml for the agent. Since the first line antifungal agent for systemic fungal infection is amphotericin B, there might be highly suspected resistance for this drug.

Published data for ketaconazole versus *C. albicans* tested by M27-A2 method or a very near equivalent one showed considerable disparities in MIC ranges and summary statistics.[35] The report by Still *et al.* indicates that 90% of 142 *C. albicans* isolates from burns patients were inhibited only at or above 16 µg/ml ketoconazole.[7] In the present study, 7% of the isolates were resistant to ketoconazole and MIC_{90} for the isolates was 0.125 µg/ml.

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Meanwhile, 1.2% of the isolates were resistant to nystatin. Nystatin is active against most *Candida* species and is most commonly used for suppression of local candidal infections. No data was available in relation to resistance to nystatin. It is too toxic for parenteral administration and is only used topically. As shown in Table 3, comparison between in-vitro antifungal susceptibility of *C. albicans* isolated in different studies revealed that the MIC_{50} and MIC_{90} for the isolates in this study against the above-mentioned antifungals are the same or different in one or two dilutions.

We conclude that an increasing rate of *C. albicans* resistance to antifungal agents may be due to the frequent use of these agents in the prophylaxis of fungal infections in immunocompromised patients. For this reason, it would be convenient to carry out antifungal susceptibility studies in order to establish the in-vitro activities of antifungal agents against local isolates and also to detect shifts toward resistance as early as possible. This study underscores the presence of *C. dubliniensis,* as reported for the first time from Iran; therefore, to consolidate the findings further investigations into geographical and epidemiological distributions seem warranted.

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