Efficacies of echinocandins versus azoles antifungal agents against *Candida albicans* and *Candida dubliniensis* isolates

Abstract

**Background:** *Candida* infection is a world wide problem with greatly changed epidemiology. *Candida dubliniensis* is an emerging non-albicans, shares several phenotypic and genotypic characteristics and closely related to *Candida albicans*. Fluconazole-resistant *Candida* spp. becomes a significant life-threatening problem especially in high risk patients. Echinocandins are promising antifungals with no recorded resistance.

This study was done, first to differentiate *C. dubliniensis* from *C. albicans* by different phenotypic and molecular methods and to compare in vitro activities of echinocandins versus azoles against *Candida* spp.

**Subjects and Methods:** One hundred fifty-seven oral and vaginal swabs collected from Dermatology Outpatients Clinic of Zagazig University Hospitals. Cultivation was done on Brilliance Candida agar. Growth at elevated temperature; tobacco agar VITECK2-YST and PCR were investigated. Antifungal susceptibility testing was done to *C. albicans* and *C. dubliniensis* to fluconazole and caspofungin by microdilution method.

**Results:** A total of 76 *Candida* isolates were identified. Most of them (61.8%) were *C. albicans*, followed by *C. glabrata*, *C. dubliniensis* and the least was *C. krusei* (14.5%), (13.2%) and (10.5%), respectively. For further differentiation between *C. albicans* and *C. dubliniensis*, PCR had the highest sensitivity (100%). However, tobacco agar, growth at elevated temperature and VITECK2-YST had (82.5%), (59.6%) and (84.2%) sensitivities, respectively. The Percentage of fluconazole-resis-
tant *C. dubliniensis* and *C. albicans* were (55.6%) and 23 (49%), respectively, and with MIC ≥64 μg/ml for both species. Both *C. albicans* and *C. dubliniensis* isolates were (100%) susceptible to caspofungin at MIC range of 0.06-1 μg/ml.

**Conclusion:** This study demonstrated that Brilliance Candida Agar is a reliable culture medium for differentiation of *Candida* spp., and caspofungin showed good activity against fluconazole-resistant *C. albicans* and *C. dubliniensis*.

**Key words:** *C. dubliniensis*, *C. albicans*, Caspofungin, Fluconazole resistance.

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**Introduction**

Invasive infections caused by *Candida* species had been increasing worldwide in last decades. The most frequently isolated *Candida* species is *C. albicans*, but non-*albicans Candida* (NAC) is increasing cause of significant morbidity and mortality in seriously ill patients [1].

Exogenous and endogenous predisposing factors render some individuals more vulnerable to infections by genus *Candida* such as HIV-infected patients, those under chemotherapy, broad-spectrum antibiotics and corticosteroids for long time, terminally ill patients with hematologic diseases, transplanted patients, and patients with diabetes mellitus [2].

*Candida* species accounts for about one-third of vulvovaginitis affecting most women [3]. *C. dubliniensis* is an emerging pathogen causing oropharyngeal, vaginal and systemic infections. Inspite the fact of being similar to *C. albicans* phenotypically, it differs from in epidemiology, certain virulent attributes and its ability to rapidly develop fluconazole resistance [4].

Phenotypic tests are useful tools for primary identification of *C. dubliniensis*, including growth at 42 and 45°C, change in carbohydrate assimilation, production of chlamydospores on tobacco agar medium. Chromogenic media such as *Candidachromogenic agar* have been developed to improve yeast identification and rapid differentiation between *C. albicans* and NAC based on colonial morphology and color production especially in mixed cultures. They are technically simple and cost effective compared to expensive and time consuming conventional method [5, 6,7]. Molecular methods permit a conclusive identification by using rapid and easy differentiation between *C. albicans* and *C. dubliniensis* [2].

Extensive use of azoles for treatment of *Candida* infection or discontinuing their use before infection is fully eliminated will result in changes the pattern of fungal susceptibility. This process will promote selection of innately resistant strains or development of secondary resistance to these drugs in a previously susceptible *C. albicans* [8, 9].

Echinocandins are fungicidal against fluconazole resistant *C. albicans*. Guidelines favor use of echinocandins as first line therapy in haemo- dynami-
cally unstable patients, because of lack of cross-resistance to azole, limited toxicity and a favorable drug-drug interactions profile [10, 11].

The objectives of this work were first to identify closely related species C. dubliniensis from C. albicans using phenotypic and molecular methods and to compare in vitro their susceptibility to both caspofungin and fluconazole.

Material and Methods

Study design

A prospective cross-sectional study was carried out over 12-month. The investigations were performed at the Microbiology and Immunology Department and Dermatology Outpatients Clinic, Faculty of Medicine, Zagazig University Hospitals. All patients with suspected fungal infections, and those with repeated or chronic infections, patients showing no response to antibiotic therapy or diabetics and patients with malignancies were included.

Ethical consideration

The study was approval by the Scientific Ethical Committee at Zagazig University Hospital and informed consent from each patient was obtained.

The following laboratory procedures were performed for all collected non-repetitive specimens from nail, skin scrapping, oral and vaginal swabs of investigated patients:

Direct film was done using lactophenol cotton blue for all specimens.

Primary isolation was done on Sabaraud’s dextrose agar and Brain heart infusion agar (Oxoid, United Kingdom) at 25°C and 37°C, respectively for primary fungal isolation.

Germ tube test

For species differentiation: Cultivation was done on Brilliance Candida agar (Oxoid, England).

Phenotypic methods

All Candida isolates were examined by growth at elevated temperature 42°C, 45°C and cultivation on Tobacco agar medium (according to Khan et al., 2004). Identification of Candida species isolates were performed by Vitek 2 ID yeast system. Vitek 2 cards were incubated at 35.5 °C for 18 h, and optical density readings were taken automatically every 15 min. A final identification of excellent, very good, good, acceptable, or low-discrimination was considered to be correct.

PCR test

The identity of C. albicans and C. dubliniensis was confirmed by PCR using the following method. Extraction of DNA from Candida isolates was performed using Genomic BYF DNA Extraction Mini Kit (INTRON, Biotechnology, Inc, Korea). Amplification was done by ready to go PCR beads Amersham Biosciences, England. The following primers (Pioneer, Germany) were used: First Primers for C. dubliniensis sense: CDU2 – 5’AGT TAC TCT TTC GGG GGT GGC CT 3’; Anti-sense: NL4CAL – 5’ AAG ATC ATT ATG CCA ACA TCC TAG GTA AA 3’). Second primer C. albicans Sense: CAL5 – 5’ TGT TGC TCT CTC GGG GGC GGC CG3’; Anti-sense: NL4CAL – 5’ AAG ATC ATT ATG CCA ACA TCC TAG GTA AA 3’). PCR cycles were run in thermal cycler according to Mannarelli et al. [12]. Conditions consisted of an initial denaturation at 94°C for 2 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds. Primer annealing at 34°C for one minute and elongation at 72°C for one minute followed by an extended elongation step at 72°C for 5 minutes. PCR product underwent agarose gel electrophoresis (2%), followed by staining with ethidium bromide solution. Amplified DNA
fragments were detected using UV transilluminator, and the size of the amplicons was estimated by comparing them with the Gene Ruler 1 Kb plus 1 kb DNA ladder (Thermo Fisher Scientific, USA).

- Antifungal susceptibility testing was done for *C. albicans* and *C. dubliniensis* isolates to both fluconazole and caspofungin [13].
- Broth microdilution testing was performed according to NCCLS guidelines document M27-A2. An inoculum concentration adjusted to (1.5 ± 1.0) × 10^3 cells/ml and RPMI 1640 medium buffered to pH 7.
- A 0.1 ml of yeast inoculum was added to each tube. Serial two-fold dilutions were adjusted. Final concentrations of the antifungal agents ranged from 0.007 to 8 μg/ml for caspofungin (Merck & Co., Whitehouse Station, Pa., USA) and from 0.12 to 128 μg/ml for fluconazole (Pfizer, Inc., New York, N.Y., USA).
- The susceptibility trays were incubated at 35°C. MIC end points were read after 48 h. Drug free and yeast free controls were included.

A quality control strain of *C. albicans* (ATCC 10231) was obtained from Pharma, Suid, Egypt, were included.

### Statistical analysis of data

The collected data were presented, summarized, tabulated & analyzed using computerized software statistical packages (EPI-info Version 6.04 & SPSS version 19 Inc. Chicago, USA), A *P*-value <0.05 was considered to be statistically significant at 95% confidence interval. Chi-square & fisher exact tests were used to compare proportions. Sensitivity = (true positive) × 100 / (true positive + false negative)

Specificity = (true negative) × 100 / (true negative + false positive)

### Results

#### Distribution and identification of *Candida* species

Cultivation and identification of the *Candida* isolates on Brilliance Candida agar revealed the following: A total of 47 (61.8%) were *C. albicans* as shown by the presence of parrot green color. Eleven isolates (14.5%) were identified as *C. glabrata* by their beige yellow colonies. Ten isolates (13.2%) as *C. dubliniensis* by their dark green colonies, and 8 (10.5%) of isolates were *C. krusei* identified by brown-pink

### Table 1. Prevalence of *Candida* species in different clinical specimens.

<table>
<thead>
<tr>
<th><em>Candida</em> species/No.</th>
<th>Vaginal (no. 59)</th>
<th>Oral (no. 17)</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td><em>C. albicans</em>/47</td>
<td>41</td>
<td>87%</td>
<td>6</td>
<td>13%</td>
</tr>
<tr>
<td><em>C. dubliniensis</em>/10</td>
<td>3</td>
<td>30%</td>
<td>7</td>
<td>70%</td>
</tr>
<tr>
<td><em>C. glabrata</em>/11</td>
<td>8</td>
<td>73%</td>
<td>3</td>
<td>27%</td>
</tr>
<tr>
<td><em>C. krusei</em>/8</td>
<td>7</td>
<td>88%</td>
<td>1</td>
<td>12%</td>
</tr>
<tr>
<td><strong>Total no.</strong></td>
<td><strong>76</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**P** value < 0.001=highly significant.
colonies (Table 1). Out of 47 C. albicans and 10 C. dubliniensis isolates, 52 isolates (91%) were positive for germ tube test, including 9 and 43 isolates from oral vaginal samples, respectively. While the rest isolates were germ tube negative. This result was statistically significant (P<0.001), regarding comparison between C. albicans and non-albicans Candida species isolates (Table 1). Regarding growth at elevated temperatures 42°C and 45°C, thermostolerance of C. albicans isolates showed, growth of 7 and 40 at the corresponding grades. While poor growth of C. dubliniensis isolates was detected with higher temperature, 9 and 1 grew at these temperatures respectively. These results showed statistically significant difference regarding growth of Candida albicans at 45°C (P value < 0.05). Culture on tobacco agar media showed 8 isolates produced orange colonies with peripheral hyphal fringe and chlamydo-spores, suggesting C. dubliniensis, while 44 of the isolates grew as white-cream-colored colonies with no hyphal fringe or chlamydo-spores suggesting C. albicans isolates. Vitek 2 compact ID-YST system identified excellently 51/ 57 (89.5%) of isolates, including 8 (14%) of C. dubliniensis (6 isolates from oral and 2 isolates from vaginal samples) and 23 (40%) of C. albicans (2 isolates from oral and 21 isolates from vaginal samples). Very good identification level was detected in 20 (35%) of C. albicans (4 isolates from oral and 16 isolates from vaginal samples), while only good identification level was detected in 3 (5%) of C. dubliniensis isolates from oral samples with acceptable low-discrimination level. Also, 3 isolates (4.3%) from vaginal sample were misidentified (Table 2).

**Antifungal susceptibility testing**

The identity of all isolates of C. albicans (47) and C. dubliniensis (10) were first confirmed by PCR. Antifungal susceptibility testing to fluconazole revealed that resistance pattern was recorded at MIC ≥ 64 μg/ml for 23 (49%) of the tested C.albicans. Another 23 (49%) of the isolates were intermediate susceptible with MIC ranged from (16-32) μg/ml, and only one isolate was full susceptible with MIC ≥ 0.5 μg/ml. A total of 50% C. dubliniensis isolates

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**Table 2. Performance comparison of different methods used for differentiation of C. albicans and C. dubliniensis**

<table>
<thead>
<tr>
<th>Identification method for tested isolates (no.=57)</th>
<th>C. albicans (no. = 47)</th>
<th>C. dubliniensis (no. = 10)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco agar</td>
<td>White-cream Colonies, no hyphal fringe or chlamydo-spores 44</td>
<td>Orange colonies with peripheral hyphal fringe 8</td>
<td>82.5%</td>
<td>91%</td>
<td>91.2%</td>
</tr>
<tr>
<td>Temperature (42°C)</td>
<td>7</td>
<td>9</td>
<td>59.6%</td>
<td>63.2%</td>
<td>71.9%</td>
</tr>
<tr>
<td>(45°C)</td>
<td>40</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VITEK 2 Ex</td>
<td>----</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VG</td>
<td>23</td>
<td>----</td>
<td>84.2%</td>
<td>84.2%</td>
<td>94.7%</td>
</tr>
<tr>
<td>G</td>
<td>20</td>
<td>----</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acc</td>
<td>----</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR band = 175 bp</td>
<td>47</td>
<td>10</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
were resistant and the remaining were intermediate susceptible (Table 3). Both Candida species were susceptible to caspofungin with MIC$_{50}$ and MIC$_{90}$ of (≥ 0.5μg/ml and ≥ 0.25μg/ml) and (≥ 1 and 0.5 μg μg/ml) for C. albicans and C. dubliniensis isolates, respectively. Quality control C. albicans strain showed MIC ≥ 0.25 μg/ml according to Tobudic et al. [14].

Discussion

The epidemiology of Candida infections has changed over the last two decades and the number of patients suffering from such infections has increased dramatically. Furthermore, non-albicans Candida (NAC) species have become more numerous than Candida albicans [15].

The present study indicated that non-albicans Candida accounted for 38.2% of all clinical isolates. Similar result was also recorded by other study where the incidence of NAC species was 30.1% [16] (Table 1). A study by Kremery and Barmes [17] found that NAC species caused 65% of all candidiasis in the general population. This variation in incidence of NAC may be attributed to differences in population types, and frequent exposure to the different antifungal agents lead to a selective pressure toward increasing resistant isolates.

The predominance of C. albicans over NAC as well as the incidence of other Candida spp. is shown in (Table 1). These results were similar to study of Burman et al. (2013), which has reported the incidence rates of C. albicans with 76.6%, followed by C. tropicalis, C. krusei, C. parapsilosis (11.7%), (6.7%) and (5%), respectively [18].

This study has also demonstrated that the majority of C. albican isolates grew better at higher temperature than C. dubliniensis, and only 15% of the isolates were unable to grow at 45°C as shown in Table 2. A study of Momani et al (2005) has demonstrated similar results [19]. Therefore, we suggest that temperature growth method can be used as screening test for initial identification of C. dubliniensis among germ tube-producing Candida strains [20].

Tobacco agar medium was described by for primary isolation of Cryptococcus neoformans [21], however, later this medium was recommended for differentiation of C. albicans and C. dubliniensis [22]. In this study, Tobacco agar medium was evaluated and it has found to be highly specific and has accuracy of 91% and 91.2% for detection of C. albicans and C. dubliniensis, respectively (Table 2). However, other study has recorded 100% accuracy of Tobacco agar as simple mean differentiating between C. dubliniensis and C. albicans [22].

**Table 3.** Antifungal susceptibility testing results of C. albicans and C. dubliniensis isolates to fluconazole.

<table>
<thead>
<tr>
<th>Candida species (No.)</th>
<th>S (MIC &lt; 8μg/ml)</th>
<th>I (MIC = 16-32μg/ml)</th>
<th>R (MIC ≥ 64μg/ml)</th>
<th>$\chi^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>C. albicans (47)</td>
<td>1</td>
<td>2%</td>
<td>23</td>
<td>49%</td>
<td>23</td>
</tr>
<tr>
<td>C. dubliniensis (10)</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>50%</td>
<td>5</td>
</tr>
</tbody>
</table>

S: sensitive R: resistant I: intermediate.
The current study showed the results of automated Vitek 2 compact ID-YST system in detection of both *C. dubliniensis* and *C. albicans* has reached 84.2% specificity and 94.7% accuracy (Table 2). Many other studies evaluated Vitek 2 compact ID-YST card and demonstrated variable identification rates (84% to 99%) between *Candida* species [23, 24]. However, Meletiadis et al. [25], observed 15% species misidentification by Vitek 2-YST among *Candida* isolates. In general, automated system might misidentified certain number of *Candida* strains which are involved in outbreaks of nosocomial infections and prevent application of necessary measurements to control their infection [25].

Culture based phenotypic identification techniques are slow and can be resulted infungal misidentification. In recent years, DNA-based methods have been developed and proved to be excellent to diagnose *Candida* infections, especially methods of PCR and RAPD with high level of sensitivity, specificity and accuracy [26].

This study has demonstrated that our used methods for *Candida* species identification were highly sensitive and specific (statistically significant (P = 0.05). Except growth at elevated temperature showed low sensitivity and specificity (59.6%) and (63.2%), respectively.

The present study showed that PCR method is a reliable molecular technique, and it has the highest sensitivity, specificity and accuracy to detect pure DNA of *C.albicans* in gel-electrophoresis with the size of 175 bp. However, PCR alone lacks the discriminatory power to differentiate between both *C. albicans* and *C. dubliniensis* strains in a mixed sample with sense and antisense primers for both types according to other study [12]. Moreover, our results gave a supportive validity and reliability to Brilliance Candida agar which showed concordant results to that obtained by PCR.

Candidal systemic infections are associated with high mortality rate as they are difficult to treat due to their intrinsically low susceptibility to many antifungal drugs including fluconazole. While caspofungin as part of echinocandins has currently more clinical use due to their solubility, antifungal spectrum, and pharmacokinetic properties; it disrupts the cell walls of *Candida* species by inhibiting β-1,3-D-glucan synthase and results in cell rupture and death. The presented MIC results of *C. albicans* isolates to fluconazole and caspofungin (Table 3 and 4) were in agreement with other studies which showed similar results obtained from 160 medical centers worldwide in association with Merck Clinical Microbiology Laboratory [13,27-28]. However, a study carried by Adhikary and Joshi [29] from South India reported different results to our as their total *Candida* isolates from various clinical samples were susceptible only 75% to fluconazole. Complete resistance of *C. dubliniensis* isolates to fluconazole is of critical importance in treatment of

**Table 4.** MIC$_{50}$ and MIC$_{90}$ of tested isolates of *C. albicans* and *C. dubliniensis* to caspofungin

<table>
<thead>
<tr>
<th>Candida species (No.)</th>
<th>MIC$_{50}$</th>
<th>MIC$_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> (47)</td>
<td>≥ 0.5 μg/ml</td>
<td>≥ 1 μg/ml</td>
</tr>
<tr>
<td><em>Candida dubliniensis</em> (10)</td>
<td>≥ 0.25 μg/ml</td>
<td>≥ 0.5 μg/ml</td>
</tr>
</tbody>
</table>
immune compromised patients with serious infections. Therefore, antifungal susceptibility testing in vitro is a recommended tool to predict the efficacy of the agent before its use in treatment.

A large multicenter studies described that caspofungin retains mostly activity against azole-resistant Candida isolates. Caspofungin has demonstrated an excellent safety profile with few serious drug-related side effects and toxicity in treatment of patients [28].

Despite their close taxonomic similarities, C. albicans and C. dubliniensis differed markedly in their antifungal responses. C. dubliniensis showed more resistance to fluconazole antifungal than that observed with C. albicans isolates [29].

In conclusion, this study demonstrated that Brillance Candida Agar is a reliable culture medium for phenotypic of Candida spp. A similar result was also observed using PCR for differentiation between Candida spp. Fluconazole-resistant C. albicans and C. dubliniensis isolates from our patients were highly susceptible to caspofungin.

There is no conflict of interest
References


