The distribution of species and susceptibility of amphotericin B and fluconazole of yeast pathogens isolated from sterile sites in Taiwan

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To study the demographic changes of yeasts causing invasive infections in Taiwan, especially with respect to species distribution and antifungal susceptibility, we analyzed isolates obtained from four sterile sites of patients in 19 hospitals in 2002 (155 strains) and again from the same hospitals in 2006 (208 strains). Blood was the most common source of the yeasts, accounting for 73.8% of the total isolates, followed by ascites (21.5%), cerebrospinal fluid (3%), and synovia (1.7%). *Candida albicans* was the most frequently recovered species (50.1% of the total), followed by *Candida tropicalis* (20.7%), *Candida glabrata* (11.6%), *Candida parapsilosis* (8.5%), *Cryptococcus neoformans* (3.9%), *Candida krusei* (0.8%), and nine other species (4.3%). There were one (0.3%) and seven (1.9%) isolates with minimum inhibitory concentrations (MICs) of amphotericin B ≥ 2 mg/l after 24 h and 48 h incubation, respectively. In addition, there were 15 (4.3%) and 31 (8.6%) isolates with MICs of fluconazole ≥64 mg/l under the same conditions. The MIC90 value of amphotericin B was 1 mg/l. The MIC90 values of fluconazole were 4 mg/l after 24 h incubation and 32 mg/l after 48 h incubation. Interestingly, MICs for fluconazole ≥64 mg/l after 24 h were significantly higher for isolates obtained in 2006 than those in 2002 after 24 h (7.1% vs. 0.7%, p = 0.009) and 48 h (13.5% vs. 2%, p = 0.0003) incubations. The demographic difference between these two surveys is mainly due to one species, *C. tropicalis*.

Keywords yeast, *Candida* species, TSARY, invasive infections, drug susceptibility

Introduction

Due to the increase in the size of the at-risk population, the prevalence of invasive fungal infections has significantly increased. *Candida* species are the most frequently isolated fungal pathogens causing morbidity and mortality in seriously immunocompromised hosts. Overall, the five most frequently isolated *Candida* species are *Candida albicans, Candida tropicalis, Candida glabrata, Candida parapsilosis*, and *Candida krusei* [1–3]. In the past, nearly 80% of candidemia could be attributed to *C. albicans* [4]. However, although *C. albicans* is still the most prominent species causing candidemia, the prevalence of non-*C. albicans Candida* species has increased [1,2,5,6].

The opportunistic *Candida* species which exist as part of commensal microflora in humans are usually the etiological agents causing infections [7]. Thus, it is important to investigate if isolates collected nationwide have distinct characteristics in connection to their sources. The recovery of the yeasts from sterile sites argues for their potential pathogenic character versus that of contaminant or colonizer. However, most available epidemiological data were related to bloodstream infections by *Candida* species [2,8,9]. Therefore, it is of interest to evaluate the role of yeasts in addition to *Candida* species from sterile sites other
Yeasts from sterile sites

than blood. Thus, we set out to determine the species distribution and drug susceptibility of yeast pathogens from four sterile sites, i.e., ascites, blood, cerebrospinal fluid (CSF), and synovial, collected in Taiwan.

Materials and methods

Organisms and media

Yeast isolates from sterile sites were collected according to previous studies [10, 11]. There were 24 and 22 hospitals participating in the surveys of Taiwan Surveillance of Antimicrobial resistance of Yeasts (TSARY) and a total of 3,926 and 3,726 yeasts recovered in 2002 and 2006, respectively. Each hospital was asked to submit all yeast pathogens isolated from sterile sites and the first 40 isolates from non-sterile sites during the period July to September in 2002 and then again in 2006. A total of 945 (2002) and 1,052 (2006) strains were frozen at −70°C in bead-containing Microbank cryovials (PRO-LAB Diagnostics, Austin, TX, USA) for storage and sent to National Health Research Institutes (NHRI) for further analyses. Among these isolates, 155 (2002) and 208 (2006) recovered from the four sterile sites noted, were analyzed further. In principle, only one isolate was accepted from each specimen per patient. Nevertheless, more than one species was isolated from 15 specimens. Furthermore, isolates from two different specimens of two patients were requested by clinicians in the contributing hospitals to be accepted in the survey. After their arrival at NHRI, these isolates were subcultured on Sabouraud dextrose agar (BBL, Becton Dickinson Cockeysville, MD, USA) to assess the purity and identification. Pure isolates were labeled and stored in vials containing 50% glycerol at −70°C awaiting further analyses.

Identification

The identifications of the isolates were reassessed in the laboratory at NHRI. The identification procedure for the yeast isolates was modified based on our previous report [12]. In 2002, isolates identified as C. albicans by participating hospitals were first subjected to the germ tube test in brain heart infusion (BBL) medium containing 10% goat serum (Gibco BRL, Grand Island, NY, USA) at 37°C for 2–3 h [13]. The VITEK Yeast Biochemical Card (YBC; bioMérieux, Hazelwood, MO, USA) was then used to identify the yeast tube negative isolates, as well as those identified as non-C. albicans Candida species by participating hospitals. In addition, ID 32C (bioMérieux) test strips were used to assess the NHRI result when the YBC showed less than 90% confidence. No Candida dubliniensis isolates were collected in the TSARY during 2002 using the high growth temperature sensitivity method for distinguishing C. albicans from C. dubliniensis. To further determine if there were C. dubliniensis isolates collected from general patients in the participating hospitals, the identification procedures of the 2006 TSARY 2006 were modified. First, all isolates were subjected to ID 32C. The YBC was then used for the identification of the isolates when ID 32C showed less than 90% confidence and for those with discrepancies in the information provided by the hospitals. The sequence of internal transcribed spacer regions (ITS) and the D1/D2 region of ribosomal DNA [14, 15] was then used for further assessment when both ID 32C and YBC failed to reach confidence greater than 90%.

Antifungal susceptibility testing

The MICs of amphotericin B and fluconazole for each isolate were determined by in vitro antifungal susceptibility testing according to the guidelines of M27-A published in 1997 by the Clinical and Laboratory Standards Institute (CLSI) [16]. The RPMI medium 1640 provided by Gibco BRL buffered with MOPS was used for the testing. Strains from American Type Culture Collection including C. albicans (ATCC 90028), C. krusei (ATCC 6258), and C. parapsilosis (ATCC 22019) were employed as the standard controls. The growth of each isolate was measured by Biotrak II plate reader (Amersham Biosciences, Biochrom Ltd., Cambridge, England) after incubation at 35°C for 24 and 48 h.

For Candida species, the MIC was defined as the concentration of drugs capable of reducing the turbidity of cells to greater than 95% (for amphotericin B) and 50% (for fluconazole) as compared to controls after incubation at 35°C. Isolates with MICs ≥2 mg/l to amphotericin B were considered to be resistant and those with MICs ≤1 mg/l were susceptible. Those isolates with MICs ≥64 mg/l for fluconazole were considered to be resistant, whereas those with MICs ≤8 mg/l were susceptible. The isolates with MICs to fluconazole in the range of 16–32 mg/l were susceptible-dose dependent. The MICs of 50% and 90% of the total population were defined as MIC<sub>50</sub> and MIC<sub>90</sub>.

Among the phenomena associated with resistance, ‘trailing’ describes the reduced but persistent growth that some isolates exhibit at drug concentrations above the MIC in broth dilution tests with azole antifungal agents, such as fluconazole [17]. Experimentally, trailing is defined when the MIC of an isolate after 48 h incubation is approximately 4-fold higher than that at the 24 h point [18].

Database and analyses

The database for this study contained the characteristic information of each submitted isolate: hospital origin,
location and type of the hospital, identification and source of the isolate. The statistical significance of the differences in frequencies and proportions was determined by the chi-square test with Yates correction.

**Results**

There were 24 and 22 hospitals participating in TSARY in 2002 and 2006, respectively. A total of 155 and 208 isolates causing invasive infections during this period recovered at the same 19 hospitals in 2002 and 2006, respectively, were subjected to further analyses for this study (Table 1). In total, there were 15 taxa which included 7 Candida species and 8 non-Candida species (Table 1). There were more species collected in 2006 than in 2002 (14 vs. 9). Of the 363 isolates, 336 (92.6%) were Candida species and C. albicans was the most frequently recovered (50.1%), followed by C. tropicalis (22.4%), C. glabrata (12.3%), C. parapsilosis (8.5%), and C. krusei (0.8%) (Table 1). In addition, C. dubliniensis was not detected to cause invasive infections in these two surveys.

Though different species had different prevalences in different body sites, blood was the most common source, accounting for 73.8% of the total isolates, followed by ascites (21.5%), CSF (3%), and synovia (1.7%) (Table 2). Of the 268 isolates causing bloodstream infections, C. albicans was still the most frequently isolated species accounting for 47.4% of the total isolates, followed by C. tropicalis (22.4%), C. glabrata (12.3%), C. parapsilosis (10.4%), and C. krusei (0.7%) (Table 2). A total of 68%, 16.7%, 9%, and 3.8% of isolates from ascites were C. albicans, C. tropicalis, C. glabrata, and C. parapsilosis, respectively. Among the 11 isolates from CSF, nine were of Cryptococcus neoformans and one each for Candida rugosa and Trichosporon asahii.

There were three isolates that failed to grow in RPMI medium. Due to the slow growth of 14 C. neoformans and one C. parapsilosis isolates, the susceptibilities to amphotericin B and fluconazole were determined after 24 and 48 h incubation for 345 (2002) and 360 isolates (2006) (Table 2). The MIC\(_{50}\) and MIC\(_{90}\) values of amphotericin B were 0.5 mg/l and 1 mg/l, respectively after either 24 h or 48 h incubation. There was only one isolate, a Cephalotheca species, with MICs for amphotericin B 2 mg/l after 24 h incubation. Among the isolates with MICs for amphotericin B ≥2 mg/l after 48 h incubation were four C. tropicalis, and one each for C. glabrata, Cephalotheca species, and Trichosporon asahii. The MIC\(_{50}\) and MIC\(_{90}\) values of fluconazole were 0.25 mg/l and 1 mg/l after 24 h incubation and 0.5 mg/l and 32 mg/l after 48 h incubation. There were 15 (4.3%) and 31 (8.6%) isolates with MICs for fluconazole ≥64 mg/l after 24 h and 48 h incubation, respectively. The four isolates of uncommon species with MICs of fluconazole ≥64 mg/l were two Rhodotorula mucilaginosa and one each for Candida guilliermondii and Pseudozyma antarctica. Interestingly, the prevalence of isolates with MICs of fluconazole ≥64 mg/l was significantly higher in 2006 than in 2002 (after 24 h incubation, 7.1% vs. 0.7%, \(p=0.009\); after 48 h incubation, 13.5% vs. 2%, \(p=0.0003\)). Furthermore, the prevalence of different species with MICs of fluconazole ≥64 mg/l was different. After 48 h incubation, all C. glabrata and C. parapsilosis isolates were susceptible to fluconazole. In contrast, there were 100% of C. krusei, 29.3% (22/75) C. tropicalis, and 1.1% (2/180) C. albicans isolates with MICs ≥64 mg/l of fluconazole. The high prevalence of C. tropicalis isolates with fluconazole MICs ≥64 mg/l in 2006 contributed to the dramatic difference in the prevalence of isolates with high MICs between the two surveys. Analyzed according to the sources, there were 33.3% (2/6), 9% (24/266), 6.5% (5/77), and 0% of isolates from synovia, blood, ascites, and CSF, respectively, with fluconazole MICs ≥64 mg/l after 48 h incubation.

**Discussion**

C. albicans is the most frequently isolated species causing candidemia (from 36.2% to 58%) [1,9,19]. Interestingly, the prevalence of non-C. albicans Candida species is significantly different in various geographic areas. Candida glabrata appears to be the most commonly recovered non-C. albicans Candida species in western countries, whereas C. tropicalis is the most common one in Asia [5,6,20,21]. Furthermore, recent reports indicate that in certain areas in Asia, C. tropicalis has surpassed C. albicans to become the most frequently isolated member of the genus [20,21].

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Table 2: Distribution and drug susceptibilities of yeast species from four sterile sites in TSABTs in 2002 and 2006

<table>
<thead>
<tr>
<th>Source</th>
<th>C. albicans</th>
<th>C. tropicalis</th>
<th>C. glabrata</th>
<th>C. parapsilosis</th>
<th>C. krusei</th>
<th>C. neoformans</th>
<th>others</th>
<th>Subtotal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>48 (62.3a)</td>
<td>79 (15.2)</td>
<td>29 (51.7)</td>
<td>31 (50)</td>
<td>100 (100)</td>
<td>1 (100)</td>
<td>1 (50)</td>
<td>121 (150)</td>
</tr>
<tr>
<td>Ascites</td>
<td>28 (36.4)</td>
<td>25 (23.8)</td>
<td>5 (31.7)</td>
<td>4 (16)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>37 (21.7)</td>
</tr>
<tr>
<td>CSF</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Synovia</td>
<td>1 (1.3)</td>
<td>1 (1)</td>
<td>2 (4.9)</td>
<td>1 (5.9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>Subtotal</td>
<td>77 (105)</td>
<td>34 (41)</td>
<td>17 (25)</td>
<td>19 (22)</td>
<td>12 (2)</td>
<td>3 (11)</td>
<td>4 (12)</td>
<td>155 (208)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug susceptibility</th>
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<tbody>
<tr>
<td>Tested isolates</td>
</tr>
<tr>
<td>Amphotericin B</td>
</tr>
<tr>
<td>&lt;2</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>Fluconazole &lt;64</td>
</tr>
<tr>
<td>≥64</td>
</tr>
<tr>
<td>48 h</td>
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<tr>
<td>Tested isolates</td>
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<tr>
<td>Amphotericin B</td>
</tr>
<tr>
<td>&lt;2</td>
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<tr>
<td>2</td>
</tr>
<tr>
<td>Fluconazole &lt;64</td>
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<tr>
<td>≥64</td>
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</tbody>
</table>

| anumber (percentage of each species %), bminimum inhibitory concentration (mg/l). |
In this study, we have found that only 33 (12.3%) C. glabrata isolates were collected from blood even though this species is the second most common one causing candidemia in the USA and The Netherlands [2,22]. Our investigation showed that even though Taiwan is on the major traffic route to western countries, it still shares the same trend as other areas in Asia in the distribution of Candida species, a condition also noted in Singapore [20]. Our data are consistent with the recent report that the prevalence of candidemia caused by C. glabrata has decreased in intensive care units of a major hospital in Taiwan [23].

There were more species collected in 2006 than in 2002 (14 vs. 9) which raises several issues about the rare species. For example, even though Cephalotheca species are black yeasts or moulds (debatable depending on one’s point of view), we have included it in our collection. Recently, Rhodotorula mucilaginosa has emerged as an opportunistic pathogen in immunocompromised patients even though it was considered to be a non-virulent contaminant in blood cultures in the past [24]. It is very likely that there will be an increasing number of unusual fungal infections concomitant with further advances in medicine, especially in areas involving immunosuppressive therapy, use of corticosteroids and antimicrobials, and widespread use of central venous catheters.

It has been reported that non-C. albicans Candida species causing candidemia have higher resistance rates to fluconazole than C. albicans. These include 35% of C. glabrata, 75% of C. krusei, 10–25% of Candida lusitaniae, and 10–25% of C. tropicalis [25,26]. Noteworthy, all C. glabrata isolates in this study were susceptible to fluconazole, which is similar to our previous report [1]. This low rate of resistance to fluconazole in this study may be explained by fewer patients having prior fluconazole treatments in Taiwan [27,28]. This is also consistent with the concept that continuous exposure to azoles seems to have an essential role in the development of resistance to fluconazole in Candida species [29].

Trailing growth refers to the phenomenon of reduced but persistent growth at antifungal drug concentrations above the MIC in broth in vitro dilution tests and may interfere with the in vivo evaluation of resistance [17,18]. Approximately one half of the 31 isolates with fluconazole MICs ≥64 mg/l after 48 h incubation exhibited the trailing phenomenon. Interestingly, this was not observed with the isolates of the four uncommon species. Recently, a good correlation between 24 h and 48 h MICs and the clinical relevance of 24 h fluconazole MICs has been reported [30]. Thus, for the common species, especially C. tropicalis, whether the CLSI guideline should change the MICs of fluconazole after 24 h instead of 48 h incubation needs further study. Another issue requiring further investigation is whether the trailing growth, especially that of C. tropicalis, affects the outcome of therapy.

In addition to 2002 and 2006, there was another TSARY survey in 1999. In all three studies, C. tropicalis was the most frequently isolated non-C. albicans Candida species [11,31,32]. Furthermore, the susceptibilities of C. tropicalis isolates to fluconazole fluctuated dramatically. For C. tropicalis isolates causing candidemia, approximately 33.3%, 0%, and 54.8% from 1999, 2002, and 2006, respectively, had MICs ≥64 mg/l of fluconazole after 48 h incubation. Recently, we have reported an association between fluconazole susceptibility and genetic relatedness among C. tropicalis isolates from TSARY 1999 based upon the results of multilocus sequence typing [33]. The data showed that DST140 was a predominant type of C. tropicalis isolates among those with MICs ≥64 mg/l of fluconazole in TSARY 1999 [34,35]. Interestingly, our recent results showed that DST140 was detected again among C. tropicalis isolates in the TSARY 2006 [36]. Spreading of closely related clonal strains of C. tropicalis may explain the decreased susceptibility of this species to fluconazole in Taiwan. Thus, whether this is an endemic problem and the conditions for their disappearance in 2002 require further investigation. It would be interesting to determine if this phenomenon also exists in other countries.

Undoubtedly, the recognition of opportunistic infections and drug resistance are still challenging issues for clinicians and medical technologists. Improvements in the identification of Candida species, as well as other unusual yeast pathogens will, in turn, contribute to the increased recognition of cases. However, a high index of suspicion teamed with newly developed techniques for culture, identification and susceptibility tests, will likely allow earlier diagnosis and the hope for better treatment.

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