



## Species distribution and drug susceptibilities of *Candida* isolates in TSARY 2010<sup>☆</sup>

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and TSARY Hospitals

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### ABSTRACT

Susceptibilities to antifungal drugs of 1083 *Candida* isolates collected in Taiwan Surveillance of Antimicrobial Resistance of Yeasts in 2010 were determined. There were 422 (39%) *C. albicans*, 270 (24.9%) *C. tropicalis*, 258 (23.8%) *C. glabrata*, 87 (8%) *C. parapsilosis*, 18 (1.7%) *C. krusei*, and 28 (2.6%) of 13 other species. In the present study, we have applied species-specific clinical breakpoints for common species and epidemiological cutoff values for rare species. We found that majority of isolates were susceptible to tested drugs. A total of 15, 3, 2, and 0 isolates were not susceptible to fluconazole, voriconazole, amphotericin B, and anidulafungin, respectively. We found that three of the four fluconazole non-susceptible *C. albicans* isolates were resistant to voriconazole. Hence, there is an issue of cross-resistance among azole-type drugs.

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### 1. Introduction

The increased prevalence of fungal infections is due to the expanding number of immunocompromised patients, increased use of invasive medical devices, and extensive use of broad spectrum antibiotics (Pappas et al., 2009; Pfaller and Diekema, 2010; Yang and Lo, 2001). Hence, the epidemiology of fungal infections has become important in the last few decades. *Candida* species are the most frequently isolated fungal pathogens responsible for significant morbidity and mortality (Chen et al., 2006; Chen et al., 2011; Cheng et al., 2004; Pfaller and Diekema, 2007; Warnock, 2007; Yang et al., 2010) and they are leading causes of fungal infections in the world (Chuang et al., 2010; Colombo et al., 2006; Edwards, 1995; Pfaller and Diekema, 2010; Ruan and Hsueh, 2009; Warnock, 2007; Wisplinghoff et al., 2004; Yang et al., 2010). Among the 30 to 40 *Candida* species causing diseases in human (Johnson, 2009; Richardson, 1991), *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. krusei* are the five most common species causing candidiasis (Almirante et al., 2005; Colombo et al., 2006; Pfaller and Diekema, 2010; Yang et al., 2010).

Azoles (fluconazole and voriconazole), echinocandins (anidulafungin, caspofungin, and micafungin), and polyene (amphotericin B) are the three classes of drugs most commonly prescribed for treating systemic fungal infections. The emergence of drug-resistant fungal pathogens is a growing concern in medical settings (Sanglard and Odds, 2002; White et al., 1998; Yang and Lo, 2001).

In 1999 and again in 2002 and 2006, three national surveys, Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY), have been conducted. The drug susceptibilities of the 632, 909, and 964 *Candida* isolates collected in 1999, 2002, and 2006 respectively, have been determined (Yang et al., 2004; Yang et al., 2005a; Yang et al., 2008). Among them, 0.5% (1999), 2.5% (2002), and 1.8% (2006) were with amphotericin B MICs  $\geq 2$  mg/L after 48-hour (h) incubation. There were 8.4%, 1.9%, and 17.1% of the isolates in 1999, 2002, and 2006, respectively, with fluconazole MICs  $\geq 64$  mg/L after 48 h (Yang et al., 2004; Yang et al., 2005a; Yang et al., 2008).

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 azoles (Arthington-Skaggs et al., 2002; Lee et al., 2004). In TSARY 2006 (Yang et al., 2008), we have found, among the isolates with fluconazole MICs  $\geq 64$  mg/L after incubation for 48 h, 72.7% (96/132) *C. tropicalis*, 70% (7/10) *C. albicans*, and 44.4% (4/9) *C. glabrata* isolates exhibited the trailing phenomenon, whereas none of the *C. krusei* isolates did. Epidemiological cutoff values for *Candida* species and species-specific clinical breakpoints for fluconazole (Pfaller et al., 2010), voriconazole (Pfaller et al., 2011a), and the echinocandins (Pfaller et al., 2011b) for common species have been established to minimize the trailing and to support a shorter time for reporting MICs. In the present study, we have conducted a follow-up TSARY in 2010. We have applied newly defined species-specific breakpoints for fluconazole, voriconazole, and echinocandins for common species and epidemiological cutoff values for the rare species (Pfaller and Diekema, 2012) to investigate drug susceptibilities of 1083 *Candida* isolates.

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## 2. Materials and methods

### 2.1. Organisms and media

Yeast isolates were collected from the 23 hospitals participating in TSARY from July 1 to September 30 in 2010 (Yang et al., 2013). Each hospital was asked to submit all yeast pathogens from sterile sites and the first ten *C. albicans* and 40 non-*C. albicans* yeast isolates from non-sterile sites. In principle, only one isolate was accepted from each specimen. Nevertheless, when there were multiple species isolated from one specimen, one isolate from each species was analyzed. All the collected isolates were stored frozen at  $-70^{\circ}\text{C}$  in vials containing 50% glycerol. After their arrival at the laboratory at National Health Research Institutes (NHRI), these isolates were first sub-cultured on sabouraud dextrose agar (SDA, BBL, Becton Dickinson Cockeysville, MD, USA) to assess the purity and identification. When there were more than one species from one frozen vial, the cells from the vial will be streaked onto CHROMagar *Candida* medium (BBL, Becton Dickinson Cockeysville, MD, USA) and pure single colony of each morphotype was labeled and stored in vials containing 50% glycerol at  $-70^{\circ}\text{C}$  awaiting further analyses.

#### 2.1.1. Identification

The identifications of the isolates were first performed by the contributing TSARY hospitals and then reassessed in the laboratory at NHRI. The procedure for the identification was established previously (Lo et al., 2001; Yang et al., 2013). In brief, all isolates identified as *C. albicans* by the TSARY hospitals were subjected to germ tube assay in RPMI medium 1640 (Gibco BRL, 31800-022) containing 10% fetal bovine serum (GibcoBRL, US-628531). The germ tube-positive isolates failing to grow at  $42^{\circ}\text{C}$  were identified by sequencing their ribosomal DNA (rDNA) (Leaw et al., 2007). All isolates identified as non-*C. albicans* by the TSARY hospitals were tested with VITEK 2 (bioMérieux, Marcy l'Etoile, France). The sequences of the internal transcribed spacer (ITS) and/or the D1/D2 regions of rDNA were used for species identification when one of the followings occurred: the identification probability of the VITEK 2 was less than 85%, the identification of an organism was inconsistent between the hospital and the NHRI laboratory, and when uncommon species were reported. The ITS regions were amplified by the primers ITS1, 5'-TCCGTAGGTGAACCTGCGG-3', and ITS4 5'-TCCTCCGTTATTGATATGC-3', and the D1/D2 regions were amplified by the primers NL1 5'-GCATATCAATAAGCGGAGAAAAG-3' and NL4 5'-GGTCCGTGTTCAA-GACGG-3' (Leaw et al., 2007). In addition, to determine whether *Candida nivariensis* isolates were recovered in the present study, we have streaked out all isolates identified as *C. glabrata* onto CHROMagar *Candida* medium (Alcoba-Florez et al., 2005b). The rDNA sequences of the cells showing white color on the CHROMagar *Candida* medium were then determined (Alcoba-Florez et al., 2005a).

#### 2.1.2. Antifungal susceptibility testing

The MICs of the four antifungal agents were determined by the *in vitro* antifungal susceptibility testing established in our laboratory (Yang et al., 2004), according to the guidelines of M27-A3 recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2008). RPMI medium 1640 (31800-022, Gibco BRL) was used for growth and dilution of the yeast. Strains from American Type Culture Collection (ATCC), including *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), and *C. parapsilosis* (ATCC 22019), were used as the standard controls. The growth of each isolate was measured by the Biotrak II plate spectrophotometric reader (Amersham Biosciences, Biochrom, Cambridge, England) after incubation at  $35^{\circ}\text{C}$  for 24 h.

Standard powders of amphotericin B, kindly provided by Bristol Myers Squibb, and anidulafungin, fluconazole, and voriconazole, by Pfizer, were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of the anidulafungin and voriconazole were from

0.0156 mg/L to 8 mg/L, amphotericin B, 0.0313–16 mg/L, and fluconazole, 0.125–64 mg/L.

The MICs were defined as the concentration of drugs capable of reducing the turbidity of cells to greater than 50% for anidulafungin, fluconazole, and voriconazole and completely inhibiting cell growth for amphotericin B. The epidemiological cutoff values for amphotericin B after 24-h incubation was 2 mg/L for all species (Pfaller et al., 2012). The newly defined species-specific breakpoints for the five common *Candida* species, *C. albicans*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* were applied in the present study (Pfaller and Diekema, 2012). For fluconazole, the clinical breakpoints of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* are as following: MICs  $\leq 2$  mg/L were considered to be susceptible,  $\geq 8$  mg/L resistant, and 4 mg/L susceptible-dose dependent (SDD); for *C. glabrata*, MICs  $\leq 32$  mg/L were SDD,  $\geq 64$  mg/L resistant. For voriconazole, the clinical breakpoints of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* were MICs  $\leq 0.125$  mg/L susceptible,  $\geq 1$  mg/L resistant, and 0.25–0.5 mg/L intermediate. For *C. krusei*, MICs  $\leq 0.5$  mg/L were susceptible,  $\geq 2$  mg/L resistant, and 1 mg/L intermediate. For anidulafungin, the clinical breakpoints of *C. albicans*, *C. krusei*, and *C. tropicalis* were MICs  $\leq 0.25$  mg/L susceptible,  $\geq 1$  mg/L resistant, and 0.5 mg/L intermediate. For *C. parapsilosis* and *C. guilliermondii*, the breakpoints were changed to MICs  $\leq 2$  mg/L susceptible,  $\geq 8$  mg/L, resistant, and 4 mg/L, intermediate. For *C. glabrata*, they were  $\leq 0.125$  mg/L susceptible,  $\geq 0.5$  mg/L resistant, and 0.25 mg/L intermediate. For the species of which clinical breakpoints have not been established, we applied epidemiological cutoff values instead (Pfaller and Diekema, 2012). The MICs of 50% and 90% of the total population were defined as MIC<sub>50</sub> and MIC<sub>90</sub>, respectively.

#### 2.1.3. Database and analysis

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 Mantel-Haenszel correction or fisher exact with 2-tailed correction.

## 3. Results

### 3.1. Distribution of *Candida* species

In the present study, we collected the first ten *C. albicans* and 40 non-*C. albicans* yeast isolates from non-sterile sites. Therefore, the prevalence of *C. albicans* was underestimated. Even so, it was still the most frequently isolated species in the present study, accounting for 39% of the total isolates. *Candida tropicalis* (270, 24.9%) and *C. glabrata* (258, 23.8%) were the two most frequently isolated non-*C. albicans* *Candida* species followed by *C. parapsilosis* (87, 8%), *C. krusei* (18, 1.7%), *C. guilliermondii* (7, 0.6%), and others (21, 1.9%) (Table 1). When classified according to the sources, there were 489 (45.2%) isolates from urine, 213 (19.7%) from blood, 141 (13%) from sputum, 53 (4.9%) from catheter tip, 40 (3.7%) from wound, 33 (3%) from ascites, 28 (2.6%) from pus, 22 (2%) from bronchial washing, and 64 (5.9%) from other 23 different sites. *Candida albicans* was still the most common cause for candidemia (103, 48.4%), followed by *C. tropicalis* (41, 19.2%), *C. parapsilosis* (35, 16.4%), *C. glabrata* (22, 10.3%), *C. krusei* (2, 0.9%), and others (10, 4.7%).

### 3.2. Susceptibilities to amphotericin B and anidulafungin of *Candida* species

The range of amphotericin B MICs of the 1083 isolates was from 0.0131 to 2 mg/L (Table 2). The MIC<sub>50</sub> and MIC<sub>90</sub> were 0.25 and 0.5 mg/L, respectively. Only two *C. albicans* isolates were with amphotericin B MIC  $\geq 2$  mg/L. The range of anidulafungin MICs was from 0.0156

**Table 1**  
Distribution of 1083 *Candida* isolates from different body sites.

	<i>C. albicans</i>	<i>C. tropicalis</i>	<i>C. glabrata</i>	<i>C. parapsilosis</i>	<i>C. krusei</i>	<i>C. guilliermondii</i>	Other 12 spp	Total
Urine	132	137 (50.7) <sup>a</sup>	200 (77.5)*	14 (16.1)*	3 (16.7)	0	3 (14.3)	489 (45.2)
Blood	103	41 (15.2)	22 (8.5)*	35 (40.2)*	2 (11.1)	2 (28.6)	8 (38.1)	213 (19.7)
Sputum	69	48 (17.8)*	11 (4.3)*	1 (1.1)*	7 (38.9)*	2 (28.6)	3 (14.3)	141 (13)
Tip	24	14 (5.2)	5 (1.9)*	7 (8)	1 (5.6)	0	2 (9.5)	53 (4.9)
Wound	17	7 (2.6)	4 (1.6)	9 (10.3)*	0	2 (28.6)	1 (4.8)	40 (3.7)
Ascites	23	3 (1.1)	6 (2.3)	0	0	0	1 (4.8)	33 (3)
Pus	17	5 (1.9)	1 (0.4)	4 (4.6)	1 (5.6)	0	0	28 (2.6)
Bronchial washing	11	7 (2.6)	2 (0.8)	0	1 (5.6)	0	1 (4.8)	22 (2)
other 23 sites	26	8 (3)	7 (2.7)	17 (19.5)	3 (16.7)	1 (14.3)	2 (9.5)	64 (5.9)
Total	422	270	258	87	18	7	21	1083

<sup>a</sup> Number of isolate (percentage in the same species).

\*  $P \leq 0.05$ , comparison of percentage for each species/source group vs. percentage in total population.

to 2 mg/L (Table 2). The MIC<sub>50</sub> and MIC<sub>90</sub> were 0.0156 and 0.0625 mg/L, respectively. All isolates were susceptible to anidulafungin.

### 3.3. Susceptibilities to azoles of *Candida* species

The range of fluconazole MICs of the 1083 isolates was from 0.125 to 64 mg/L (Table 2). The MIC<sub>50</sub> and MIC<sub>90</sub> were 0.25 and 1 mg/L, respectively. There were two *C. albicans* and one *C. parapsilosis* with MIC  $\geq 8$  mg/L and two *C. glabrata* with MICs  $\geq 64$  mg/L, considered as resistant. The MIC<sub>50</sub> and MIC<sub>90</sub> of some species, such as *C. glabrata* (1 and 4 mg/L), *C. krusei* (8 and 16 mg/L), and *C. guilliermondii* (1 and 4 mg/L) were higher than others. The range of MICs to voriconazole was from 0.0125 to 8 mg/L (Table 2). The MIC<sub>50</sub> and MIC<sub>90</sub> were 0.0156 and 0.0313 mg/L, respectively. There were three *C. albicans* with 8 mg/L voriconazole MICs, considered as resistant.

## 4. Discussion

In the present study, we have determined the distribution of species and susceptibilities to antifungal drugs of 1083 *Candida*

isolates collected in TSARY 2010. The distribution of species according to body sites in the present study was similar to that of the three previous surveys in 1999, 2002, and 2006 (Yang et al., 2004; Yang et al., 2005a; Yang et al., 2008). Interestingly, the ratio of isolates from blood in the present study was higher than that in the TSARY surveys in 2002 (13.9%  $P = 0.0006$ ) (Yang et al., 2005a) and 2006 (15%  $P = 0.006$ ) (Yang et al., 2008). *Candida albicans* was still the most common cause for candidemia (48.4%) in the present study, consistent with other reports (Cheng et al., 2004; Peman et al., 2005; Pfaller and Diekema, 2007; Yang et al., 2010). The distribution of *C. parapsilosis* in human body sites was different from other species because it has a broader distribution in the environment and can be isolated from various non-human sources (Abi-Said et al., 1997; Hedderwick et al., 2000; Levin et al., 1998). In the present study, there were 87 isolates of *C. parapsilosis*, about 8% of the 1083 isolates. However, they comprised 16.4% of the 213 blood isolates, consistent with our previous report (Yang et al., 2005a). A major proportion (40.2%) of those 87 isolates was from blood, which may have resulted from their capability to adhere to catheters. Overall, *C. parapsilosis* has accounted for 3% to 27% of the fungemia cases in other studies (Pfaller et al., 1999; Pfaller et al.,

**Table 2**  
Antifungal susceptibilities of 1083 *Candida* spp.

MIC (mg/L)	<i>C. albicans</i>	<i>C. tropicalis</i>	<i>C. glabrata</i>	<i>C. parapsilosis</i>	<i>C. krusei</i>	<i>C. guilliermondii</i>	Others	Total
Total	422	270	258	87	18	7	21	1083
Amphotericin B								
0.5	402	264	236	86	17	7	20	1032
1	18	6	22	1	1	0	1	49
2	2	0	0	0	0	0	0	2
MIC <sub>50</sub>	0.25	0.25	0.5	0.25	0.25	0.125	0.125	0.25
MIC <sub>90</sub>	0.5	0.5	0.5	0.25	0.5	0.5	0.25	0.5
Anidulafungin								
$\leq 0.125$	422	270	256	11	18	1	18	996
0.25	0	0	2	9	0	1	2	14
0.5	0	0	0	42	0	3	1	46
1	0	0	0	20	0	2	0	22
$\geq 2$	0	0	0	5	0	0	0	5
MIC <sub>50</sub>	0.0156	0.0156	0.0313	0.5	0.0313	0.5	0.0313	0.0156
MIC <sub>90</sub>	0.0156	0.0313	0.0313	1	0.0625	1	0.25	0.0625
Fluconazole								
$\leq 2$	418	269	232	86	1	6	17	1029
4	2*	1*	15	0	0	1*	4*	23
8	0	0	0	1*	9	0	0	10
16	2 <sup>#</sup>	0	7	0	8	0	0	17
32	0	0	2*	0	0	0	0	2
$\geq 64$	0	0	2 <sup>#</sup>	0	0	0	0	2
MIC <sub>50</sub>	0.125	0.25	1	0.25	8	1	0.5	0.25
MIC <sub>90</sub>	0.25	0.25	4	0.5	16	4	4	1
Voriconazole								
$\leq 0.125$	419	270	248	87	18	7	21	1070
0.25	0	0	8	0	0	0	0	8
1	3 <sup>#</sup>	0	2	0	0	0	0	5
MIC <sub>50</sub>	0.0156	0.0156	0.0156	0.0156	0.125	0.0313	0.0156	0.0156
MIC <sub>90</sub>	0.0156	0.0313	0.0313	0.0156	0.125	0.0625	0.0625	0.0313

\*Susceptible-dose dependent; #Resistant.



2000; Warnock, 2007; Yang et al., 2004). The majority of *C. glabrata* (77.5%) was isolated from urine samples, consistent with previous reports that *C. glabrata* is second only to *C. albicans* as a cause of candiduria (Yang et al., 2006; Yang et al., 2011).

According to the new breakpoints, majority of isolates collected in TSARY 2010 were susceptible to all tested drugs. A total of 15, 3, and 2 isolates were not susceptible to fluconazole, voriconazole, and amphotericin B, respectively. Previously, we have found an association between fluconazole susceptibility and genetic relatedness among *C. tropicalis* isolates (Chou et al., 2007; Wang et al., 2007). We have reported that two dominant *C. tropicalis* clones, DST140 and DST98, with fluconazole MICs  $\geq 64$  mg/L circulated in Taiwan (Li et al., 2009). Recently, we have found that genetically related *C. tropicalis* with fluconazole MICs  $\geq 64$  mg/L were isolated from the human hosts and environmental samples (Yang et al., 2012). In the present study, of the 270 *C. tropicalis* isolates, none was resistant to fluconazole and only one (0.4%) was SDD. Thus, we are in the process of genotyping the *C. tropicalis* isolates collected in TSARY 2010 to further assess the association of DST140 and DST98 with fluconazole resistance.

When comparisons were made among species, there were distinct variations in the fluconazole susceptibility. Only one *C. krusei* was with MICs  $< 8$  mg/L. In contrast, only one *C. parapsilosis* was with MICs  $\geq 8$  mg/L. These results were consistent with previous reports that *C. krusei* had high and *C. parapsilosis* had low fluconazole-resistant rates (Akova et al., 1991; Pfaller et al., 2000; Pfaller et al., 2004; Yang et al., 2005b; Yang et al., 2008). It has been reported that continuous exposure to azoles seems to have a major impact on selecting fluconazole-resistant *Candida* species, especially *C. glabrata* (Kontoyiannis, 2002). In the present study, 1.6% of the *C. glabrata* isolates were with fluconazole MICs  $\geq 32$  mg/L. Therefore, our previous observation that low proportion of patients receiving prior fluconazole treatments (Cheng et al., 2005) may explain the low fluconazole-resistant *C. glabrata* in Taiwan than other areas (Fleck et al., 2007; Pfaller and Diekema, 2007).

In the present study, we found that three of the four isolates with fluconazole MICs  $\geq 4$  mg/L were resistant to voriconazole. As reported previously (Yang et al., 2004a), this finding emphasizes the important issue of cross-resistance phenomenon among azole drugs.

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