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Susceptibilities to amphotericin B and fluconazole of *Candida* species in Taiwan Surveillance of Antimicrobial Resistance of Yeasts 2006

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Abstract

Susceptibilities to amphotericin B and fluconazole of 964 *Candida* isolates collected in Taiwan Surveillance of Antimicrobial Resistance of Yeasts in 2006 were determined. There were 419 (43.5%) *Candida albicans*, 246 (25.5%) *Candida tropicalis*, 211 (21.9%) *Candida glabrata*, 62 (6.4%) *Candida parapsilosis*, 14 (1.5%) *Candida krusei*, and 12 (1.2%) others. Interestingly, 16 of the 17 amphotericin B-resistant isolates were non-albicans *Candida* species. The resistant rate to amphotericin B has decreased from 2.5% in 2002 to 1.8% in 2006. On the other hand, there were 132 *C. tropicalis*, 14 *C. krusei*, 10 *C. albicans*, and 9 *C. glabrata* isolates that had MICs to fluconazole ≥64 μg/mL. The prevalence of isolates with such high MICs was significantly higher than that in 2002 (17.1% versus 1.9%). Our results further indicate that most of the isolates with MICs to fluconazole ≥64 μg/mL exhibited the "trailing" phenomenon.

Keywords: *Candida*; Susceptibility; Resistance

1. Introduction

Because of alterations in immune status and invasive hospital procedures (White et al., 1998; Yang and Lo, 2001), infections caused by opportunistic pathogens, such as yeasts, are becoming important causes of morbidity and mortality in immunocompromised patients. In the past 2 decades, nosocomial yeast infections have increased significantly worldwide. For example, the prevalence of nosocomial candidemia increased 27-fold from 1981 through 1993 at a major hospital in Taiwan (Chen et al., 1997; Hung et al., 1996). In the United States, yeast infection also ranks as the 4th most common cause of nosocomial bloodstream infection (Wisplinghoff et al., 2004). Several antifungal drugs have been applied to render the situation, and as a result of broad prophylactic usages and long-term treatments with those drugs, the prevalence of drug resistance has become an important issue in various yeast infections, which have profound effects on human health (Marr et al., 2001; Pfaller et al., 2003; Yang et al., 2004b).

Candida species have various degrees of susceptibility to frequently used antifungal drugs. For instance, *Candida lusitaniae* is relatively resistant to amphotericin B (Hadfield et al., 1987). *Candida krusei* is intrinsically resistant to fluconazole, and *Candida glabrata* is less susceptible or has a higher MICs to it than other *Candida* species (Akova et al., 1991; Orozco et al., 1998; Yang et al., 2004b). This phenomenon illustrates the importance of identification and surveillance of *Candida* species in the clinical settings.

In 1999 and again in 2002, 2 national surveys in Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY) have been conducted. The drug susceptibilities of the 632 and 909 isolates collected in 1999 and 2002, respectively, have been determined (Yang et al., 2004b, 2005b). Among them, 0.5% (1999) and 2.5% (2002) were resistant to

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amphotericin B. There were 8.4% of the isolates in 1999 that had MICs to fluconazole ≥64 μg/mL, whereas only 1.9% in 2002 (Yang et al., 2004b, 2005b). In 2006, a follow-up survey was taken place, and 964 *Candida* isolates were collected and analyzed. The article is to report the result of the susceptibilities to antifungal drugs of *Candida* species in TSARY 2006 and the trends of the resistance in Taiwan from 1999 to 2006.

2. Materials and methods

2.1. Organisms and media

Yeast isolates were collected according to previous studies (Lo et al., 2001; Yang et al., 2005b) from the 22 hospitals participating in TSARY from July to September in 2006. Each hospital was asked to submit all yeast pathogens from blood and the first 10 *Candida albicans* and 40 non-albicans *Candida* species isolates from nonsterile sites. In principle, only 1 isolate was accepted from each specimen. Nevertheless, when there were multiple species isolated from 1 specimen, 1 isolate from each species was analyzed. All the collected isolates were stored frozen at −70 °C in bead-containing Microbank cryovials (Pro-Lab Diagnostics, Austin, TX). After their arrival at the laboratory at National Health Research Institutes (NHRI), Taiwan, ROC, these isolates were 1st subcultured on Sabouraud dextrose agar (Becton Dickinson, Cockeysville, MD) to assess the purity and identification. Pure isolates were labeled and stored in vials containing 50% glycerol at −70 °C awaiting further analyses.

2.2. Identification

The identifications of the isolates were reassessed in the laboratory at the NHRI. The identification procedure for the yeast isolates was modified based on our previous report (Lo et al., 2001). All isolates were subjected to API-32C (bioMérieux, St. Louis, MO). The VITEK Yeast Biochemical Card (YBC, bioMérieux) would then be used for the identification of the isolates when the API-32C showed less than 90% confidence and for the isolates whose information was inconsistent to that provided by the hospitals. The sequence of internal transcribed spaces of ribosomal DNA (Leaw et al., 2006, 2007) was applied for further assessment when both API-32C and YBC failed.

2.3. Antifungal susceptibility testing

The MICs to amphotericin B and to fluconazole of each isolate were determined by the same in vitro antifungal susceptibility testing as those in TSARY 1999 and TSARY 2002 (Yang et al., 2004b, 2005b), according to the guidelines of M27A published in 1997 by the Clinical and Laboratory Standards Institute (CLSI, 1997). The RPMI medium 1640 (31800-022) provided by Gibco BRL was used for the testing. Strains from American Type Culture Collection including *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), and *Candida parapsilosis* (ATCC 22019) were used as the standard controls. The final growth of each isolate was measured by Biotrak II plate reader (Amersham Biosciences, Cambridge, UK) after incubation at 35 °C for 48 h. The MICs of some isolates were also measured by Etest (AB BIODISK, Solna, Sweden) to assess the results of the broth microdilution method.

The MICs to amphotericin B and to fluconazole were defined as the MICs of drugs capable of reducing the turbidity of cells to greater than 95% and 50%, respectively. For susceptibility to amphotericin B, isolates with MIC ≥2 μg/mL were considered to be resistant, and those with MIC ≤1 μg/mL were susceptible. For susceptibility to fluconazole, isolates with MIC ≥64 μg/mL were considered to be resistant, whereas those with MIC ≤8 μg/mL were susceptible. Isolates with MICs falling in between (16–32 μg/mL) were susceptible dose dependent. The MICs of 50% and 90% of the total population were defined as MIC_{50} and MIC_{90}, respectively.

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 (Lee et al., 2004). When the MIC of an isolate measured after 48-h incubation is approximately 4-fold higher than that at the 24-h point (Arthington-Skaggs et al., 2002), the isolate is defined to have trailing growth.

2.4. Database and analysis

The database for this study contained the characteristic information of each submitted isolate: hospital origin, location and type of the hospital, and identification and source of the isolate. The statistic significance of the differences in frequencies and proportions was determined by the χ^2 test with Yates' correction.

3. Results and discussion

3.1. Distribution of Candida species

The distribution of *Candida* species in TSARY 2006 (Table 1) was similar to that of 2 previous surveys in 1999 and 2002 (Yang et al., 2004b, 2005a). *C. albicans* was the most frequently isolated species, accounting for 43.5% of the total isolates. *Candida tropicalis* (25.5%) and *C. glabrata* (21.9%) were the 2 most frequently isolated non-albicans *Candida* species, followed by *C. parapsilosis* (6.4%), *C. krusei* (1.5%), and others (1.2%). When classified according to the sources (Table 1), there were 406 (42.1%) isolates from urine, 158 (16.4%) from sputum, 145 (15%) from blood, 43 (4.5%) from wound, 40 (4.1%) from pus, 40 (4.1%) from catheter tip, 38 (4%) from ascites, and 94 (9.8%) from other 45 different sites.

Different *Candida* species had various prevalence in different body sites. Although *C. albicans* was still the most

Table 1
The distribution of *Candida* species from different sources

Sources	*C. albicans*	*C. tropicalis*	*C. glabrata*	*C. parapsilosis*	*C. krusei*	Others	Total
Urine	129	109	145	13	5	5	406 (42.1)[a]
Sputum	70	65	19	2	2	0	158 (16.4)
Blood	82	32	20	9	1	1	145 (15.1)
Wound	21	6	6	5	1	4	43 (4.5)
Pus	20	9	2	6	2	1	40 (4.1)
Tip[b]	20	13	3	4	0	0	40 (4.1)
Ascites[c]	24	7	4	2	1	0	38 (3.9)
Others	53	5	12	21	2	1	94 (9.8)
Total	419 (43.5)	246 (25.5)	211 (21.9)	62 (6.4)	14 (1.5)	12 (1.2)	964

[a] Number of isolates (percentage).
[b] Catheter tips.
[c] Body fluid, not urinary ascites or pus.

common single species (56.6%) causing candidemia in our study as well as others' (Cheng et al., 2004; Peman et al., 2005; Pfaller et al., 2007a; Yang et al., 2006), the prevalence of non-albicans *Candida* species had increased. Though *C. tropicalis* was the most common non-albicans *Candida* species in our collection, *C. glabrata* was the most common one from urine (145/277, 52.3%). The majority of *C. glabrata* (68.7%) was isolated from urine samples, consistent with the previous reports that *C. glabrata* is 2nd only to *C. albicans* as a cause of candiduria (Pfaller et al., 1999; Yang et al., 2004b).

3.2. Susceptibilities to amphotericin B of Candida species

The range of MICs to amphotericin B of the 964 isolates was from 0.125 to 2 μg/mL (Table 2). *C. krusei* was less susceptible to amphotericin B than any other species because the MIC_{50} of this species was 1 μg/mL. The overall resistant rate to amphotericin B has decreased from 2.5% in TSARY 2002 (Yang et al., 2005b) to 1.8% in TSARY 2006. In recent years, fungal infections caused by non-albicans *Candida* species have increased dramatically (Abi-Said et al., 1997; Slavin et al., 1995; Walsh et al., 2004). The phenomenon was also reflected in the distribution of resistant isolates in others' (Slavin et al., 1995; Walsh et al., 1998) as well as our studies. All 3 amphotericin B-resistant isolates in TSARY 1999 were non-albicans *Candida* species (Yang et al., 2004b). Furthermore, 20 of the 23 amphotericin B-resistant isolates in TSARY 2002 were also non-albicans *Candida* species (Yang et al., 2005b). Of the 17 amphotericin B-resistant isolates in TSARY 2006, 16 were non-albicans *Candida* species. They consisted of 12 *C. tropicalis*, 2 *C. krusei*, and 1 each of *C. glabrata* and *Candida curvatus*.

The prevalence of amphotericin B resistance of *C. krusei* (14.3%) or *C. tropicalis* (4.9%) was significantly higher than that of *C. glabrata* (0.5%) or *C. albicans* (0.2%) ($P < 0.05$). Among the frequently isolated non-albicans *Candida* species, no amphotericin B-resistant isolate of *C. parapsilosis* was detected in this study (Table 2), as well as in the 2 previous TSARYs (Yang et al., 2004b, 2005b). A total of 2, 2, and 5 *C. lusitaniae* were collected in TSARY 1999, 2002, and 2006, respectively. Up-to-date amphotericin B resistance of *C. lusitaniae* is not an issue for concern since all 9 *C. lusitaniae* was susceptible to amphotericin B. The MIC_{50} and MIC_{90} of those isolates were 0.25 and 1 μg/mL, respectively.

3.3. Susceptibilities to fluconazole of Candida species from different sources

For the susceptibility to fluconazole, a total of 756 (78.4%), 43 (4.5%), and 165 (17.1%) isolates had MICs ≤8, 16 to 32, and ≥64 μg/mL, respectively. The MIC_{50} and MIC_{90} of these isolates were 1 and 64 μg/mL, respectively. Interestingly, among the isolates with MICs ≥64 μg/mL, those from sputum (27.2%) appear to have a higher ratio than

Table 2
The susceptibility to amphotericin B of *Candida* species

MICs (μg/mL)	*C. albicans*	*C. tropicalis*	*C. glabrata*	*C. parapsilosis*	*C. krusei*	Others	Total
0.125	16 (3.8)[a]	0	4 (1.9)	0	0	1 (8.3)	21 (2.2)
0.25	213 (50.8)	45 (18.3)	49 (23.2)	23 (37.1)	0	6 (50)	336 (34.8)
0.5	134 (31.9)	126 (51.2)	113 (53.6)	29 (46.8)	5 (35.7)	2 (16.7)	409 (42.4)
1	55 (13.1)	63 (25.6)	44 (20.8)	10 (16.1)	7 (50)	2 (16.7)	181 (18.8)
2	1 (0.2)	12 (4.9)	1 (0.5)	0	2 (14.3)	1 (8.3)	17 (1.8)
Total	419	246	211	62	14	12	964
MIC_{50}	0.25	0.5	0.5	0.5	1	0.25	0.5
MIC_{90}	1	1	1	1	2	1	1

[a] Number of isolates (percentage).

those from ascites (7.9%), wound (11.6%), blood (13.1%), and urine (17.7%) ($P = 0.05$) (Table 3).

When comparisons were made among species, there were distinct variations in the fluconazole susceptibility. Of the 5 common *Candida* species, all *C. krusei* had MICs ≥64 μg/mL. In contrast, none of the *C. parapsilosis* did. These results were consistent with previous reports (Akova et al., 1991; Pfaller et al., 2000, 2004; Yang et al., 2004a, 2005b). Furthermore, susceptibilities among different species from blood also varied. All *C. albicans*, *C. glabrata*, and *C. parapsilosis* were susceptible to fluconazole, whereas 18 of the 32 (56.3%) *C. tropicalis* isolates had MICs ≥64 μg/mL (Table 3).

Interestingly, *C. glabrata* having MICs to fluconazole ≥64 μg/mL was lower than what has been reported in other geologic areas (Fleck et al., 2007; Pfaller et al., 2007b; St Germain et al., 2001). In the 3 TSARYs, the portions of isolates having MICs ≥64 μg/mL were 8.3%, 1.6%, and 4.3% isolates from 1999, 2002, and 2006, respectively. It has been reported that continuous exposure to azoles seems to have a major impact on developing resistance to fluconazole in *Candida* species, especially for *C. glabrata* (Kontoyiannis, 2002). Therefore, our previous observation that only 13% of the candidemia 92 patients had prior fluconazole treatments (Cheng et al., 2004) may explain the low fluconazole-resistant *C. glabrata* in Taiwan.

3.4. Trailing growth

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 (Lee et al., 2004). Trailing may interfere the observation of resistance level in vivo (Arthington-Skaggs et al., 2002). Thus, we have also determined whether those 165 isolates with MICs ≥64 μg/mL to fluconazole exhibit

Table 3
The susceptibility to fluconazole of *Candida* species from different sources

	MICs (μg/mL)	*C. albicans*	*C. tropicalis*	*C. glabrata*	*C. parapsilosis*	*C. krusei*	Others	Total
Urine	≦8	123 (95.3)[a]	53 (48.6)	117 (80.7)	13 (100)	0	5 (100)	311 (76.6)
	16–32	0	1 (0.9)	22 (15.2)	0	0	0	23 (5.7)
	≧64	6 (4.7)	55 (50.5)	6 (4.1)	0	5 (100)	0	72 (17.7)
	Subtotal	129	109	145	13	5	5	406
Sputum	≦8	69 (98.6)	25 (38.5)	13 (68.4)	2 (100)	0	0	109 (69)
	16–32	1 (1.4)	0	5 (26.3)	0	0	0	6 (3.8)
	≧64	0	40 (61.5)	1 (5.3)	0	2 (100)	0	43 (27.2)
	Subtotal	70	65	19	2	2	0	158
Blood	≦8	82 (100)	10 (31.2)	16 (80)	9 (100)	0	1 (100)	118 (81.4)
	16–32	0	4 (12.5)	4 (20)	0	0	0	8 (5.5)
	≧64	0	18 (56.3)	0	0	1 (100)	0	19 (13.1)
	Subtotal	82	32	20	9	1	1	145
Wound	≦8	21 (100)	2 (3.3)	5 (83.3)	5 (100)	0	3 (75)	36 (83.7)
	16–32	0	0	1 (16.7)	0	0	1 (25)	2 (4.7)
	≧64	0	4 (66.7)	0	0	1 (100)	0	5 (11.6)
	Subtotal	21	6	6	5	1	4	43
Pus	≦8	19 (95)	4 (44.4)	2 (100)	6 (100)	0	1 (100)	32 (80)
	16–32	0	0	0	0	0	0	0
	≧64	1 (5)	5 (55.6)	0	0	2 (100)	0	8 (20)
	Subtotal	20	9	2	6	2	1	40
Tip[b]	≦8	18 (90)	7 (53.8)	2 (66.7)	4 (100)	0	0	31 (77.5)
	16–32	0	1 (7.7)	0	0	0	0	1 (2.5)
	≧64	2 (10)	5 (38.5)	1 (33.3)	0	0	0	8 (20)
	Subtotal	20	13	3	4	0	0	40
Ascites[c]	≦8	24 (100)	5 (71.4)	4 (100)	2 (100)	0	0	35 (92.1)
	16–32	0	0	0	0	0	0	0
	≧64	0	2 (28.6)	0	0	1 (100)	0	3 (7.9)
	Subtotal	24	7	4	2	1	0	38
Others	≦8	51 (96.2)	2 (40)	10 (83.4)	20 (95.2)	0.0	1 (100)	84 (89.4)
	16–32	1 (1.9)	0.0	1 (8.3)	1 (4.8)	0.0	0.0	3 (3.2)
	≧64	1 (1.9)	3 (30)	1 (8.3)	0	2 (100)	0	7 (7.4)
	Subtotal	53	5	12	21	2	1	94
All	≦8	407 (97.1)	108 (43.9)	169 (80.1)	61 (98.4)	0	11 (91.7)	756 (78.4)
	16–32	2 (0.5)	6 (2.4)	33 (15.6)	1 (1.6)	0	1 (8.3)	43 (4.5)
	≧64	10 (2.4)	132 (53.7)	9 (4.3)	0	14 (100)	0	165 (17.1)
	Total	419	246	211	62	14	12	964

[a] Number of isolates (percentage).
[b] Catheter tips.
[c] Body fluid, not urinary ascites or pus.

Table 4
The distribution of trailing growth of *Candida* species with MICs greater than 64 μg/mL

Sources	*C. albicans*		*C. glabrata*		*C. krusei*		*C. tropicalis*		Subtotal		Total
	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	
Urine	3	3	5	1	5	0	13	42	26	46	72
Sputum	0	0	0	1	2	0	14	26	16	27	43
Blood	0	0	0	0	1	0	6	12	7	12	19
Pus	0	1	0	0	2	0	0	5	2	6	8
Ascites	0	0	0	0	1	0	0	2	1	2	3
Wound	0	0	0	0	1	0	0	4	1	4	5
Tip	0	1	0	0	0	0	0	4	0	5	5
Others	0	2	0	2	1	0	3	1	4	5	9
Total	3	7	5	4	14	0	36	96	58	107	165

trailing, and the results are summarized in Table 4. High percentage (64.8%) of those isolates indeed exhibited the trailing phenomenon. When classified according to species, 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.

3.5. Conclusion

In TSARY 1999, there were 14.7% *C. tropicalis* isolates having MICs ≥64 μg/mL (Yang et al., 2004b). In contrast, none of the 244 *C. tropicalis* from TSARY 2002 had MICs ≥64 μg/mL (Yang et al., 2005b). Surprisingly, in TSARY 2006, among the 246 *C. tropicalis* isolates, 132 (53.7%) had MICs ≥64 μg/mL. Recently, we have reported an association between fluconazole susceptibility and genetic relatedness among *C. tropicalis* isolates from TSARY 1999 (Chou et al., 2007; Wang et al., 2007). The DST140 was a predominant type of *C. tropicalis* isolates among those having MICs ≥64 μg/mL in TSARY 1999 (Chou et al., 2007). Furthermore, none of the 17 tested fluconazole-susceptible *C. tropicalis* isolates collected from TSARY 2002 were DST140 type (Chou et al., 2007). Interestingly, our preliminary result showed that DST140 was detected again among *C. tropicalis* isolates collected in TSARY 2006. This result may explain the dramatic fluctuation in fluconazole susceptibility among 3 TSARYs. Whether this is an endemic problem requires further investigation.

The increasing rate of reduced susceptibility to fluconazole in *C. tropicalis* has considerable clinical importance, because this species is 1 of the most frequently isolated non-albicans *Candida* species (Cheng et al., 2004; Hung et al., 2005; Lo et al., 2005; Pfaller et al., 2000; Prasad et al., 1999). Furthermore, *C. tropicalis* develops drug resistance in the presence of fluconazole much more rapidly than *C. albicans* (Barchiesi et al., 2000; Calvet et al., 1997). In addition, *C. krusei* isolates with high MICs are also closely related (Chou et al., 2007; Wang et al., 2007). These findings may explain why, to fluconazole, *C. tropicalis* has a higher rate to have MICs ≥64 μg/mL than *C. albicans*. Here we have identified that 72.7% (96/132) *C. tropicalis* exhibited the trailing phenomenon by a definition that the MIC of an isolate after 48-h incubation is 4-fold higher than that at the 24-h point. Nevertheless, whether the remaining 36 (14.6%) *C. tropicalis* isolates are "truly resistant" requires further investigation.

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