Research article

The trend of susceptibilities to amphotericin B and fluconazole of Candida species from 1999 to 2002 in Taiwan

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Abstract

**Background:** Candida species have various degrees of susceptibility to common antifungal drugs. The extent of resistance to amphotericin B and fluconazole of Candida glabrata isolates causing candidemia has been reported. Active surveillance may help us to monitor the trend of susceptibility to antifungal drugs and to determine if there is an emerging co-resistance to both drugs of Candida species, specifically, of C. glabrata in Taiwan.

**Methods:** The susceptibilities to amphotericin B and fluconazole of Candida species collected in 1999 and 2002 of the Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY) were determined by the microdilution method.

**Results:** The antifungal susceptibilities of 342 and 456 isolates collected from 11 hospitals participating in both TSARY 1999 and TSARY 2002, respectively, have been determined. The resistance rate to amphotericin B has increased from 0.3% in the TSARY1999 to 2.2% in the TSARY 2002. In contrast, the resistance rate to fluconazole has decreased from 8.8% to 2.2%. Nevertheless, significantly more C. glabrata isolates were not susceptible to fluconazole in the TSARY 2002 (47.4%) than that in the TSARY 1999 (20.8%). There were 9.8% and 11% of C. glabrata isolates having susceptible-dose dependent and resistant phenotype to fluconazole in the TSARY 1999, verse 45.3% and 2.1% in the TSARY 2002.

**Conclusion:** There was an increase of resistance rate to amphotericin B in C. glabrata. On the other hand, although the resistance rate to fluconazole has decreased, almost half of C. glabrata isolates were not susceptible to this drug. Hence, continuous monitoring the emerging of co-resistance to both amphotericin B and fluconazole of Candida species, specifically, of C. glabrata, will be an important early-warning system.

Background

In the past decade, nosocomial yeast infections have increased globally. In Taiwan, the prevalence of nosocomial candidemia increased 16-fold from 1981 through
1993 [1,2]. In the United States, yeast infections rank as the fourth most common cause of nosocomial bloodstream infection [3,4]. Furthermore, candidemia contributes considerable mortality (31% to 38%), extend the length of hospital stay [5,6], and increase social cost due to lost productivity and disabling complications [7]. Consequently, the Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY) was initiated in 1999 for epidemiological study of yeast infections in Taiwan [8,9]

*Candida* species have various degrees of susceptibility to common antifungal agents. *Candida lusitaniae* is less susceptible to amphotericin B [10] while *Candida krusei* and *Candida glabrata* are less susceptible to fluconazole than other *Candida* species [11-14]. The extent of fluconazole resistance of *C. glabrata* isolates causing candidemia has been reported throughout the United States [15]. Furthermore, *C. glabrata* exhibits variable cross-resistance to the other triazoles, such as voriconazole and posaconazole [13,16-18] and amphotericin B became the next choice.

**Methods**

**Organisms and media**

Yeast isolates were collected from 11 hospitals participating in both TSARY 1999 and TSARY 2002 [9,19]. Isolates were stored frozen at -70°C in bead containing Microbank cryovials (PRO-LAB Diagnostics, Austin, TX, USA). At the end of the collection period, isolates were kept frozen and transported by an express delivery company to the laboratory at National Health Research Institutes (NHRI) within 24 hours. After their arrival, the isolates were first sub-cultured on to sabouraud dextrose agar (SDA, BBL, Becton Dickinson Cockeysville, MD, USA) to check for purity and identifications. Pure isolates were labeled and stored in vials containing 50% glycerol at -70°C for subsequent analyses.

**Identification**

The identification procedure of yeast isolates in the NHRI laboratory was performed as described previously [8]. In general, isolates identified as *C. albicans* by hospitals were first subjected to the germ tube assay in brain heart infusion (BHI, BBL) medium containing 10% fetal bovine serum (JR12003, JRH Biosciences, Australia) at 37°C for 2–3 hours [20]. Isolates positive in germ tube assay were checked for growth at 42°C to differentiate *C. albicans* from *C. dubliniensis* [21]. The VITEK Yeast Biochemical

| Table 1: The Susceptibilities of *Candida* Species to Amphotericin B |
|---------------------|--------|--------|--------|--------|--------|--------|
| TSARY 1999          | cal    | ctr    | cgl    | cpa    | ckr    | Others | Total  |
| MIC µg/ml           |        |        |        |        |        |        |        |
| ≥ 0.25              | 19 (14.7) | 5 (5.1) | 5 (6.1) | 5 (22.7) | 0     | 3 (42.9) | 37 (10.8) |
| 0.5                 | 81 (62.8) | 57 (58.2) | 52 (63.4) | 8 (36.4) | 1 (25) | 2 (28.6) | 201 (58.8) |
| 1                   | 29 (22.5) | 36 (36.7) | 25 (30.5) | 9 (40.9) | 2 (50) | 2 (28.6) | 103 (30.1) |
| 2                   | 0       | 0       | 0       | 0       | 1 (25) | 0       | 1 (0.3)   |
| Total               | 129     | 98      | 82      | 22      | 4      | 7       | 342      |
| MIC₅₀ µg/ml          | 0.5     | 0.5     | 0.5     | 0.5     | 1.0    | 0.5     | 1.0      |
| MIC₉₀ µg/ml          | 1.0     | 1.0     | 1.0     | 1.0     | 2.0    | 1.0     | 1.0      |

| TSARY 2002          | cal    | ctr    | cgl    | cpa    | ckr    | Others | Total  |
|---------------------|--------|--------|--------|--------|--------|--------|
| MIC µg/ml           |        |        |        |        |        |        |        |
| ≥ 0.25              | 8 (4.2) | 1 (0.8) | 0      | 3 (8.3) | 0     | 0      | 12 (2.6) |
| 0.5                 | 122 (64.9) | 70 (54.2) | 17 (17.9) | 17 (47.2) | 1 (20) | 1 (33.3) | 228 (50) |
| 1                   | 56 (29.8) | 57 (44.2) | 75 (78.9) | 16 (44.5) | 0     | 2 (66.7) | 206 (45.2) |
| 2                   | 2 (1.1) | 1 (0.8) | 3 (3.2) | 0      | 4 (80) | 0      | 10 (2.2)  |
| Total               | 188     | 129     | 95      | 36     | 5      | 3      | 456      |
| MIC₅₀ µg/ml          | 0.5     | 0.5     | 1       | 0.5    | 2      | 1      | 0.5      |
| MIC₉₀ µg/ml          | 1       | 1       | 1       | 1      | 2      | 1      | 1        |

cal, *C. albicans*; ctr, *C. tropicalis*; cgl, *C. glabrata*; cpa, *C. parapsilosis*; ckr, *C. krusei*

*number of isolates (%)*
Card (YBC, bioMerieux, St. Louis, MI, USA) was then used to analyze isolates appearing to be negative by the germ tube assay in the NHRI laboratory and isolates identified as non-albicans Candida species by the hospital. API-32C (bioMerieux) was used to assess the NHRI result when the VITEK-YBC showed less than 90% confidence.

Antifungal susceptibility testing
The minimum inhibitory concentration (MIC) to amphotericin B or fluconazole of each yeast isolate was determined by in vitro antifungal susceptibility testing according to the guidelines by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) [22]. The RPMI medium 1640 (31800-022, Invitrogen Corporation, Carlsbad, CA, USA) was used for dilution. Several strains from American Type Culture Collection, namely, ATCC 14053 C. albicans, ATCC 9003 C. glabrata, ATCC 6258 C. krusei, and ATCC20019 Candida parapsilosis were used as controls. The growth of each isolate was measured by a Spectra MAX Plus (Molecular Devices Corp. Sunnyvale, California, USA) after 48-hour incubation at 35 °C. We also measured the MICs of some randomly-sampled isolates by Etest (AB Biodisk Solna, Sweden) to confirm our results by microdilution.

The interpretation of MICs was conducted according to the guidelines of the CLSI. The MICs to amphotericin B and fluconazole were defined as the lowest concentration of amphotericin B and fluconazole to reduce the turbidity of cells to greater than 95% and 50%, respectively. For amphotericin B, isolates with MIC ≥ 2 µg/ml were considered to be resistant, whereas those with MIC ≤ 1 µg/ml were susceptible. For fluconazole, isolates with MIC ≥ 64 µg/ml were considered resistant, while those with MIC ≤ 8 µg/ml were susceptible. Isolates with MICs between 16 and 32 µg/ml were susceptible-dose dependent. The MICs of 50% and 90% of the total population were defined as MIC50 and MIC90. For any species with less than ten, the MIC50 and MIC90 were not showed.

Database and analysis
The database for this study contained the following characteristic information of each submitted isolate: hospital origin, location and type of the hospital, identification and source of the isolate. The statistic significance of the differences in frequencies and proportions was determined by the chi-square test with Yates’ correction. A p value of ≤ 0.05 was considered statistically significant.

Results
Distribution of Candida species
The distribution of Candida species was similar in both surveys. Candida albicans was the most common species consisting 37.7% of the total isolates in the TSARY 1999 and 41.2% in the TSARY 2002. Candida tropicalis (28.7% in 1999 vs. 28.3% in 2002) and C. glabrata (24% in 1999 vs. 20.8% in 2002) were the two most common non-albicans Candida species, followed by C. parapsilosis (6.4% in 1999 vs. 7.9% in 2002), C. krusei (1.2% in 1999 vs. 1.1% in 2002), and others (2% in 1999 vs. 0.7% in 2002). When classified according to the sources, isolates from urine, sputum, blood, wound, and others were 143
The susceptibilities to amphotericin B are shown in Table 1. A total of 10 isolates (2.2%) were resistant to amphotericin B in the TSARY 2002, whereas only one (0.3%) in the TSARY 1999 (p < 0.05). Of these 11 amphotericin B resistant isolates, 9 were non-albicans Candida species, including 5 C. kruzei, 3 C. glabrata, and 1 C. tropicalis. In general, C. kruzei was less susceptible to amphotericin B than other species.

The susceptibilities to fluconazole of Candida species are shown in Table 2. In the TSARY 1999, a total of 289 (85.5%), 55 (12%), and 10 (2.2%) isolates were susceptible, susceptible-dose dependent, and resistant to fluconazole, respectively, whereas in the TSARY 2002, there were 391 (85.5%), 55 (12%), and 10 (2.2%). The MIC50 and MIC90 of these isolates in the TSARY 1999 were 2 µg/ml and 16 µg/ml, respectively, and in the TSARY 2002, they were 1 µg/ml and 16 µg/ml. In the TSARY 1999, 12 (12.2%) C. tropicalis, 9 (11%) C. glabrata, 5 (3.9%) C. albicans, and 4 (100%) C. kruzei, while in the TSARY 2002, 4 (2.1%) C. albicans, 3 (60%) C. kruzei, and 2 (2.1%) C. glabrata were resistant to fluconazole. Fewer isolates in the TSARY 2002 were resistant to fluconazole than that in the TSARY 1999 (p < 0.05). In contrast, more isolates from the TSARY 2002 were susceptible-dose dependent than that in the TSARY 1999 (p < 0.05). Consequently, there were similar portions of isolates susceptible to fluconazole in both surveys. Nevertheless, there were less isolates with MICs ≤ 2 µg/ml to fluconazole in the TSARY 1999 (71.6%, 207/289) than in the TSARY 2002 (81.8%, 320/391) (p < 0.05). Finally, in the TSARY 1999, 82 (24%) of isolates had MICs between 4 and 8 µg/ml to fluconazole. It was down to 71 (15.6%) in the TSARY 2002.

Discussion

The trend of susceptibilities to antifungal drugs of Candida species from 1999 to 2002 has been determined in this study. As expected, C. kruzei had the highest resistance rate to fluconazole among Candida species tested, which is consistent with previous reports [9,11]. In contrast, all C. parapsilosis isolates were susceptible to fluconazole, which is also consistent with previous reports that C. parapsilosis is the most susceptible species to fluconazole [9,18,23,24]. Though the overall resistance rate to fluconazole has decreased from 8.8% to 2.2%, there were significantly more C. glabrata isolates not susceptible to fluconazole in the TSARY 2002 than that in the TSARY 1999. Overexpression of CgCDR1, CgCDR2, and CgSNQ2-encoded efflux pumps has been shown to be a major mechanism contributing to the drug resistance [25-27]. It would be interesting to investigate the molecular mechanisms of drug resistance of those clinical resistant isolates.

Recently, triazoles have been developed as the new savior to the issue of drug resistance in Candida infection. Nevertheless, C. glabrata exhibits variable cross-resistance among triazoles [9,18,23]. Thus, amphotericin B appears to be the choice for treating systemic infections caused by this species. However, along with the increased use of amphotericin B, 20% and 36% of C. glabrata isolates from North America and Latin America, respectively, were reported to be resistant [23]. These data suggest that cross-resistance to amphotericin B and fluconazole of C. glabrata species may become a problem for clinical therapy worldwide. In our study, we found only three C. glabrata isolates resistant to amphotericin B, which is lower than what has been reported. In that study, 20% of C. glabrata causing candidemia collected in Taiwan in 2003 were resistant to amphotericin B [16]. Coincidently, more C. glabrata isolates in the TSARY 2002 (78.9%) had the MICs of amphotericin B at 1 µg/ml than that in the TSARY 1999 (30.5%). Hence, periodic surveillance is needed to closely monitor the trends of susceptibility to antifungal drugs and for early detection of the newly emerging co-resistance to amphotericin B and fluconazole of Candida species, especially, of C. glabrata.

Abbreviations used

TSARY, Taiwan Surveillance of Antimicrobial Resistance of Yeasts; NHRI, National Health Research Institutes; SDA, sabouraud dextrose agar; BHI, brain heart infusion; YBC, Yeast Biochemical Card; MIC, minimum inhibitory concentration; NCCLS, National Committee of Clinical Laboratory Standards; CLSI, Clinical and Laboratory Standards Institute.

Competing interests

The author(s) declare that they have no competing interests.

Authors’ contributions

LYL and HJL design the study and drafted the manuscript. HHC conduct the experiments with contribution with SYL. TSARY Hospitals provided isolates.

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