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The distribution and drug susceptibilities of clinical *Candida* species in TSARY 2014

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ABSTRACT

The species distribution and drug susceptibilities of 1106 *Candida* isolates collected in Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY) in 2014 were determined. *Candida albicans* is still the dominant species, accounting for 35.9%, followed by 28.3% *C. glabrata*, 26.6% *C. tropicalis*, 5.2% *C. parapsilosis*, 1.0% *C. krusei*, and 3.0% of 13 other species. Interestingly, the prevalence of candidemia caused by *C. glabrata* in the present study is significantly higher than that in previous three surveys (39/220 vs. 54/471, $P = 0.025$). We found that 31 (2.8%), 24 (2.2%), 1 (0.09%), and 0 isolates were resistant to fluconazole, voriconazole, anidulafungin, and amphotericin B, respectively. There is a significant increase in fluconazole ($P = 0.00002$) and voriconazole ($P = 0.00006$) resistant rates when compared to the isolates collected in 2010. Importantly, all the 24 voriconazole resistant isolates identified were also resistant to fluconazole. Hence, cross-resistance among azole-type drugs is an emerging issue for managing fungal infections.

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1. Introduction

Nowadays, fungal infections pose a serious threat to aging populations and immune-suppressed patients worldwide. Individuals who have weakened immune systems are the most vulnerable to fungal infections (Hsu et al., 2015; Jain et al., 2010; Kauffman, 2001; NOVAK and PLEŠKO, 2016; Park et al., 2009; Richardson, 2005). Due to the expanding number of immunocompromised patients, increased use of invasive medical devices, and extensive use of broad spectrum antibiotics (Pappas et al., 2016; Pfaller and Diekema, 2010; Yang and Lo, 2001), the prevalence of fungal infections increased significantly. Consequently, the epidemiology of fungal infections has become more important in recent decades (Bitar et al., 2014; Nucci et al., 2010; Shimodaira et al., 2012; Yoon et al., 2014). *Candida* species is a leading etiological cause of both local and systemic fungal infections, and is associated with significant morbidity and mortality. There are over 20 *Candida* species that can cause candidiasis in humans, the five most common of which are *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (Almirante et al., 2005; Colombo et al., 2006; Guinea, 2014; Orasch et al., 2014; Pfaller and Diekema, 2010; Yang et al., 2010).

Azoles (fluconazole and voriconazole), echinocandins (anidulafungin, caspofungin, and micafungin), and polyene (amphotericin B) are the three major 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 (Perlin et al., 2015; Sanglard, 2016; Sanglard and Odds, 2002; White et al., 1998; Yang and Lo, 2001).

To monitor the trends of species distribution and drug susceptibility of clinical yeast pathogens, we initiated a national survey, Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY), in 1999. Up to date, five TSARYs have been conducted. The species distribution and drug susceptibilities of the *Candida* isolates collected in 1999, 2002, 2006 and 2010 respectively, have been reported (Yang et al., 2013; Yang et al., 2010; Yang et al., 2005; Yang et al., 2008). Among them, 0.5% (1999), 2.5% (2002), and 1.8% (2006) were with amphotericin B MICs ≥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 ≥64 mg/L after 48-h (Yang et al., 2004b; Yang et al., 2005; Yang et al., 2008). Due to the trailing growth issue (Arthington-Skaggs et al., 2002; Pfaller et al., 2011; Revankar et al., 1998; Zomorodian et al., 2016) and newly defined species-specific breakpoints for common *Candida* species and epidemiological cutoff values for the rare species to investigate drug susceptibilities (Pfaller and Diekema, 2012), we applied the new breakpoints in the study of TSARY 2010. We reported that 5 (0.5%), 3 (0.3%), and 2 (0.2%) isolates characterized in TSARY 2010 were resistant to fluconazole, voriconazole and amphotericin B

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after 24-h incubation, respectively (Yang et al., 2013). In the present study, we characterized 1106 *Candida* isolates collected in 2014 and determined the trends of species distribution and drug resistance in Taiwan. These epidemiological data can be utilized as a fundamental resource to establish and evaluate management policies for fungal infections in Taiwan.

2. Materials and methods

2.1. Organisms and media

Yeast isolates were collected from the 24 hospitals participating in TSARY from June to October in 2014 according to the criteria in previous survey (Yang et al., 2005). The Research Ethics Committee certificate number for the approval of this multicenter study is EC1030509-E. We followed the same isolation collection criteria established in 2002. In brief, each hospital was asked to submit all yeast pathogens from sterile sites and up to 10 *C. albicans* and 40 non-*C. albicans* yeast isolates with clinical significance from non-sterile sites. In principle, only one isolate was accepted from each specimen. Nevertheless, when there were multiple species isolated from one specimen or multiple specimens obtained from same patient, only the last recovered isolate of same species obtained from the same body site of individual patient was analyzed. All the collected isolates were stored frozen at −70 °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), and would then be streaked onto CHROMagar *Candida* medium (BBL, Becton Dickinson Cockeysville, MD, USA) to assess the purity and identification. When there were more than one species from one frozen vial, pure single colony of each morphotype was labeled and stored in vials containing 50% glycerol at −70 °C awaiting further analyses.

2.2. Identification

The identifications of the isolates were first performed by the contributing TSARY hospitals and then reassessed in the laboratory at NHRI. There were 14, 9, 8, 8, 7 hospitals used CHROMagar *Candida*, API system, Cornmeal agar, germ tube assay, and VITEK system, respectively, at different steps for species identification. At NHRI, all the isolates were streaked onto CHROMagar *Candida* medium incubating at 35 °C for two days to ensure their purity. When the colony color of an isolate matches the *C. albicans*, *C. glabrata*, *C. krusei* or *C. tropicalis* as stated by the contributing hospital, it will be identified accordingly without further analysis conducted. The sequences of the internal transcribed spacer (ITS) and/or the D1/D2 regions of rDNA were used for species identification if any of the following occurred: isolates were identified as non-*C. albicans*, non-*C. glabrata*, non-*C. krusei*, and non-*C. tropicalis* by the hospitals; the color of colonies on CHROMagar *Candida* medium did not perfectly match the identifications provided by the hospitals; or mixed species were detected on CHROMagar *Candida* medium. In total, 259 isolates were subjected for rDNA sequencing. The internal transcribed spacer (ITS) region was amplified by the primers ITS1, 5′-TCCGTAGGTGAACCTGCGG-3, and ITS4 5′-TCCTCCGCTTATTGATATGC-3′, and/or the D1/D2 region of rDNA was amplified by the primers NL1 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and NL4 5′-GGTCCGTGTTTCAAGACGG-3′. The sequencing was performed by DNA Sequencing Core Laboratory at National Health Research Institutes. It was performed on ABI 3730XL DNA Analyzer according to Sanger dideoxy chain-termination method using ABI BigDye terminator v3.1 cycle sequencing kit. Only when the level of sequence identity was greater than 99.5% to a reported species, was the isolate identified. Thus, 2 isolates were not able to be identified to species level and labeled as *Candida* spp.

2.3. 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., 2013), according to the guidelines of M27-A3 recommended by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2008). RPMI medium 1640 (31,800–022, 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 Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific Inc., USA) after incubation at 35 °C for 24 h.

Standard powders of amphotericin B, kindly provided by Bristol Myers Squibb, and fluconazole, voriconazole, and anidulafungin, by Pfizer, were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of fluconazole were from 0.125–64 mg/L, anidulafungin and voriconazole, 0.0156–8 mg/L, and amphotericin B, 0.0313–16 mg/L.

The MICs were defined as the concentration of drugs capable of reducing the turbidity of cells to greater than 50% for fluconazole, voriconazole and anidulafungin. For amphotericin B, it means complete inhibition of cell growth. The epidemiological cutoff values for amphotericin B after 24-h incubation was 2 mg/L for all species (Pfaller and Diekema, 2012). The newly defined species-specific breakpoints for the five common *Candida* species, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* were applied in the present study (Clinical and Laboratory Standards Institute, 2012). For fluconazole, the clinical breakpoints of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* are as following: MICs ≤2 mg/L were considered to be susceptible, ≥8 mg/L resistant, and 4 mg/L susceptible-dose dependent (SDD). For *C. glabrata*, MICs ≤32 mg/L were SDD, ≥64 mg/L resistant. There is no breakpoint interpretation for *C. krusei* since it is assumed to be intrinsically resistant to fluconazole. For voriconazole, the clinical breakpoints of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* were MICs ≤0.12 mg/L susceptible, ≥1 mg/L resistant, and 0.25–0.5 mg/L intermediate. For *C. krusei*, MICs ≤0.5 mg/L were susceptible, ≥2 mg/L resistant, and 1 mg/L SDD. The breakpoints for *C. glabrata* have not been determined. For anidulafungin, the clinical breakpoints of *C. albicans*, *C. tropicalis*, and *C. krusei* were MICs ≤0.25 mg/L susceptible, ≥1 mg/L resistant, and 0.5 mg/L intermediate. For *C. parapsilosis* and *C. guilliermondii*, the breakpoints were changed to MICs ≤2 mg/L susceptible, ≥8 mg/L resistant, and 4 mg/L intermediate. For *C. glabrata*, they were ≤0.12 mg/L susceptible, ≥0.5 mg/L resistant, and 0.25 mg/L intermediate. For the species of which clinical breakpoints have not been established, we applied those of *C. albicans*. The MICs of 50% and 90% of the total population were defined as MIC_{50} and MIC_{90}, respectively. In order to further investigate the drug susceptibilities of isolates causing candidemia, we classified the isolates into blood and others groups.

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, 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. A P value less than 0.05 was considered significant.

3. Results

3.1. Distribution of Candida species

In the survey of TSARY 2014, hospitals were asked to submit the first 10 *C. albicans* and 40 non-*C. albicans* yeast isolates from non-sterile sites. Therefore, the prevalence of *C. albicans* was underestimated. Even so, it

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