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Species distribution and fluconazole susceptibility of *Candida* clinical isolates in a medical center in 2002

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Fluconazole disk-diffusion susceptibility was evaluated in 230 blood isolates and 344 non-blood clinical isolates of *Candida* spp. collected in 2002 at National Taiwan University Hospital. Up to 93.5% of blood isolates were susceptible to fluconazole, 3% were susceptible dose-dependent, and 3.5% were resistant. The minimum inhibitory concentrations at which 50% of tested isolates were inhibited (MIC_{50}) of fluconazole against *Candida* blood isolates were highest for *Candida glabrata* (5 μg/mL), followed by *Candida tropicalis* (2.4 μg/mL), *Candida albicans* (2.4 μg/mL), and *Candida parapsilosis* (0.41 μg/mL). *C. glabrata* had less fluconazole-susceptible strains (76.7%) than *C. albicans* (98.2%), *C. tropicalis* (98%) and *C. parapsilosis* (93.8%) [$p< 0.05$]. The proportions of fluconazole resistance in the non-blood isolates of *C. albicans, C. glabrata* and *C. parapsilosis* were similar to those of the blood isolates. However, the proportions of fluconazole resistance in the non-blood isolates of *C. tropicalis* surpassed those of the blood isolates (14.7% vs 2%, $p<0.05$). Comparison of species distribution of *Candida* blood isolates obtained in 2002 to those in 1981-2000 demonstrated that *C. albicans* remained the leading pathogen, and the proportion of *C. albicans* in blood isolates was lowest in 1996 (38%) and did not change significantly thereafter. However, the proportion of *C. tropicalis* increased from 14% during 1981-1993 to 22-23% during 1996-2002. Overall, the MIC_{50}, MIC_{90} and the proportion of *Candida* blood isolates with fluconazole resistance remained stable during 1994-2002.

Key words: *Candida*, fluconazole, microbial drug resistance, microbial sensitivity tests

Candida spp. are the leading pathogens of nosocomial blood stream infections at National Taiwan University Hospital (NTUH) [1,2]. Fluconazole is an effective and well tolerated antifungal agent with both parenteral and oral forms. Recently, advances in clinical microbiology have enabled detection and definition of azole resistance [3,4]. Despite an increase in the incidence of nosocomial fungal infection and increased consumption of fluconazole from 1994 through 2000, there was no significant change in the susceptibility to fluconazole for blood stream isolates of *Candida* spp. [5,6]. However, the emergence of fluconazole resistance might have been underestimated in previous studies when only blood isolates were tested. As the majority of candidemias arise from endogenous flora, isolation of *Candida* spp. from specimens other than blood, although not diagnostic, has some predictive value for invasive candidiasis [7,8]. Thus, it is important to assess fluconazole susceptibility of *Candida* non-blood isolates. In this study, fluconazole susceptibility of both blood isolates and non-blood isolates collected in 2002 at NTUH was determined and compared to results of previous studies from Taiwan [1,5].

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Materials and Methods

Candida isolates

Blood samples were cultured by inoculation into BACTEC fungal medium (Becton-Dickinson Microbiology Systems, Cockeysville, MD, USA) and tested daily for microbial growth using the BACTEC 9240 system (BD Biosciences, Sparks, MD, USA). Organisms were identified by germ tube analysis and morphology on cornmeal-Tween 90 agar [9] or, when necessary, by standard biochemical testing with the API 20C system (API BioMerieux Vitek, Inc., Hazelwood, MO, USA). Clinical isolates of *Candida* spp. were collected randomly weekly from January 1, 2002 through December 31, 2002. All the isolates were

kept at -70°C and were subcultured at least twice on Sabouraud dextrose agar (BBL, Becton-Dickinson) at 35°C prior to being tested. If the same species was found in the same culture site during a 7-day period, only the first isolate was tested.

Antifungal susceptibility testing

The minimum inhibitory concentrations (MICs) of fluconazole (Pfizer Pharmaceuticals, Inc., NY, USA) were determined by the disk-diffusion method as described previously [5,10]. This system uses a 25-μg fluconazole disk (Becton-Dickinson) and Mueller-Hinton agar supplemented with 2% glucose and 0.5 mg/L of methylene blue. Inocula were adjusted to a 0.5 McFarland density standard. Plates were incubated aerobically at 35°C for 18 to 24 hours and read by electronic image-analysis and interpreted and recorded with a BIOMIC Plate Reader System (Giles Scientific Inc., Santa Barbara, CA, USA). Zone inhibition interpretive criteria for fluconazole disc testing was based on zone diameters correlated with National Committee for Clinical Laboratory Standards (NCCLS) recommended category breakpoints for the reference macrobroth dilution method. Fluconazole breakpoints were: susceptible (≤8 mg/L or ≥19 mm), susceptible-dose dependent (16-32 mg/L or 13-18 mm), and resistant (≥64 mg/L or ≤12 mm). Quality controls were performed with each batch of clinical isolates by testing *C. albicans* ATCC 90028 with a recommended acceptable performance range of 32-43 mm. The MIC_{50} and MIC_{90} were the MIC levels at which 50% and 90% of the isolates tested were inhibited.

Statistical analyses

Statistical analyses were with the performed with Statistical Package for the Social Sciences (SPSS, version 10.0) for Windows (SPSS Inc., Chicago, IL, USA). Univariate analysis of categorical variables was done with the chi-squared test or Fisher's exact test. Continuous variables were analyzed by the Student's *t* test. All *p* values were 2-tailed, and a *p* value <0.05 was considered to indicate statistical significance.

Results

From January 1, 2002 through December 31, 2002, a total of 230 blood isolates and 344 non-blood isolates were evaluated. There were 282 isolates of *C. albicans*, 118 isolates of *C. tropicalis*, 107 isolates of *C. glabrata*, 49 isolates of *C. parapsilosis*, 9 isolates of *C. guilliermondii* and 9 *C. krusei*. The mean inhibition zone was largest for *C. parapsilosis* (32.8 ± 7.4 mm), followed by *C. albicans* (28.8 ± 6.5 mm), *C. tropicalis* (27.0 ± 8.0 mm), *C. guilliermondii* (24.7 ± 7.2 mm), *C. glabrata* (23.4 ± 7.2 mm) and *C. krusei* (18.0 ± 8.57 mm). The proportion of fluconazole resistance is shown in Fig. 1 and Table 1. The percentage of fluconazole-resistant strains was highest in *C. krusei* (55.6%), followed by *C. glabrata* (12.1%), *C. guilliermondii* (11.1%), *C. tropicalis* (9.3%), *C. albicans* (2.8%), and C. *parapsilosis* (2.0%).

Among the 230 blood isolates tested, *C. albicans* was the leading species (48.3%), followed by *C. tropicalis* (21.7%), *C. parapsilosis* (13.9%) and *C. glabrata* (13%). Only 3 isolates of *C. krusei* and 4 isolates of *C. guilliermondii* were collected during the study period. The intensive care unit (33%), medical ward (24%) and hematology/oncology ward (20%) were the leading 3 areas from which *Candida* blood isolates were collected. The proportion of fluconazole resistance in blood isolates was relatively low (3.5%). Among medically important *Candida* spp., the MIC_{50} of fluconazole against *Candida* blood isolates was highest for *C. krusei* (>64 μg/mL), followed by *C. glabrata* (5 μg/mL), *C. guilliermondii* (3 μg/mL), *C. tropicalis* (2.4 μg/mL), *C. albicans* (2.4 μg/mL), and *C. parapsilosis* (0.41 μg/mL). *C. glabrata* (76.7 %) and *C. krusei* (33.3%) had fewer fluconazole-susceptible strains than *C. guilliermondii* (100%), *C. albicans* (98.2%), *C. tropicalis* (98%), and *C. parapsilosis* (93.8%) [$p<0.05$]. On the other hand, there was no significant difference between the proportions of fluconazole resistance of blood isolates obtained from different wards.

The proportion of fluconazole resistance in blood isolates and non-blood isolates are compared in Fig. 1. The proportions of fluconazole resistance in the non-blood isolates of *C. albicans, C. glabrata* and *C. parapsilosis* were similar to those of blood isolates. However, the proportion of fluconazole resistance in the non-blood isolates of *C. tropicalis* surpassed that of blood isolates (14.7% vs 2%, $p<0.05$). The proportion of fluconazole resistance of non-blood isolates varied among different types of specimens (Table 1). The highest proportion of fluconazole-resistant strains of *C. albicans* was in specimens from the lower respiratory tract and the urinary tract.

Comparison of species distribution of *Candida* blood isolates obtained in 2002 (the present study) to those obtained during 1981-2000 [1,5] showed that *C. albicans* remained the leading pathogen, and that the proportion of *C. albicans* in blood isolates was lowest in 1996 (38%), and did not change significantly

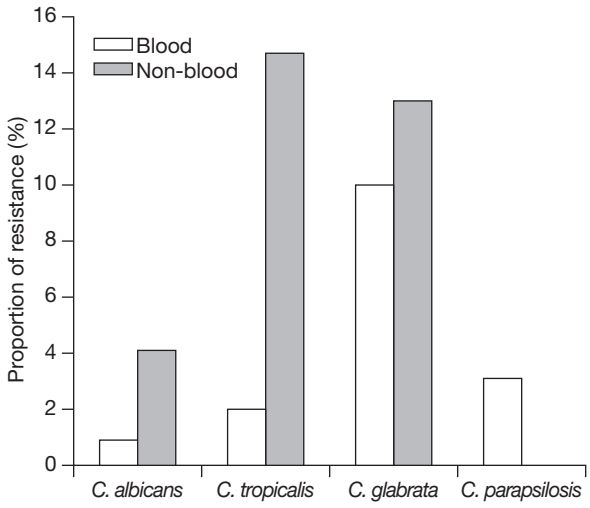

Fig. 1. Comparison of fluconazole resistance in blood isolates and in non-blood isolates of *Candida* spp., National Taiwan University Hospital, 2002.

thereafter (48% in 2002) [Table 2]. *C. tropicalis* was the second most common species. However, the proportion of *C. tropicalis* increased from 14% during 1981-1993 [1] to 22-23% during 1996-2002 [5]. Comparison of the MIC_{50}, MIC_{90} and the proportion of fluconazole resistance of *Candida* blood isolates in different study periods remained stable [5]. However, the proportion of fluconazole resistance in blood isolates was higher in 2002 than that during 1994-2000 [5] (3.5% vs 0.7%, respectively). The MIC_{50} and MIC_{90} of *C. tropicalis* blood isolates did not change significantly during 1994-2002 (Table 3) [11]. However, the MIC_{90} of *C. tropicalis* in non-blood isolates was higher than that of blood isolates (>64 μg/mL vs 10.7 μg/mL).

Discussion

The NCCLS has published a standardized broth dilution method (M27-A) for antifungal susceptibility testing of yeast [12]. Disk-based susceptibility testing is cost-effective and correlates with the reference broth methods for fluconazole [5,13-16]. To make antifungal susceptibility testing more readily available to clinical laboratories, the NCCLS Subcommittee on Antifungal Susceptibility Testing has recently developed proposed guidelines for a disk-diffusion method for testing *Candida* spp. to fluconazole (M44-P) [17]. Zone interpretive criteria (breakpoints) have been approved for fluconazole. One significant advantage of this method is that qualitative results can usually be determined after only 20 to 24 hours incubation as opposed to 48 hours with the NCCLS document (M27-A) [17]. Thus, the disk-diffusion method was used to determine fluconazole susceptibility of 574 clinical isolates collected in 2002. The present study showed that fluconazole resistance in *Candida* blood isolates remained relatively low. There was no significant difference in fluconazole resistance between blood and non-blood isolates. Thus, fluconazole resistance in clinical isolates was unlikely to have been underestimated in previous studies when only blood isolates were tested [5].

The present study demonstrated that the percentage of fluconazole resistance in blood isolates remains relatively low. However, it is slightly higher than that in the past 10 years [5]. The proportion of *C. glabrata* blood isolates collected in 2002 showed little change from previous years. Previous fluconazole use and severe

Table 1. Comparison of fluconazole resistance by specimen type and *Candida* spp.

Specimen type	*C. albicans*		*C. tropicalis*		*C. parapsilosis*		*C. glabrata*		*C. krusei*		*C. guilliermondii*		Total	
	No. of isolates	R (%)	No. of isolates	R (%)	No. of isolates	R (%)	No. of isolates	R (%)	No. of isolates	R (%)	No. of isolates	R (%)	No. of isolates	R (%)
Blood isolates	111	0.9	50	2	32	3.1	30	10	3	66.7	4	0	230	3.5
Total non-blood isolates	171	4.1	68	14.7	17	0	77	13.0	6	50	5	20	344	9.0
Non-blood sterile fluids[a]	15	0	5	0.0	0	0	5	0	1	100	1	100	27	7.4
Lower respiratory tract	57	8.8	27	7.4	1	0	15	6.5	2	100	0	0	103	9.7
Upper respiratory tract	40	0	5	20.0	0	0	3	33.3	1	0	0	0	49	4.1
Urinary tract	26	7.7	15	20.0	4	0	43	19.0	0	0	0	0	87	14.9
Other	33	0	16	25.0	12	11	11	0	2	0	4	0	78	5.1

Abbreviation: R = resistant

[a]Non-blood sterile fluids included ascites, pleural effusion, and cerebrospinal fluid.

Table 2. Trends in species distribution of *Candida* blood isolates at National Taiwan University Hospital

Study period	Distribution of *Candida* (%)					Reference
	C. albicans	*C. tropicalis*	*C. glabrata*	*C. parapsilosis*	*C. krusei*	
1981-1993	67	14	8	8	1	[1]
1996	38	23	19	17	1	[5]
2000	49	23	9	15	1	[5]
2002	48	22	13	14	1	Present study

Table 3. In vitro activities of fluconazole against isolates of *Candida tropicalis* in different study periods at National Taiwan University Hospital

Study period	Specimens	No. of isolates	MIC (μg/mL)[a]			Reference (method)
			Range	MIC_{50}	MIC_{90}	
1994-1995	Blood	33	≤0.125-4	0.5	1.0	[11] (Broth microdilution method; incubation, 24 hours)
			≤0.125-64	2	16	[11] (Broth microdilution method; incubation, 48 hours)
1994-1995	Blood	43	0.25-20.0	1.07	16.0	[5] (Disk-diffusion method)
1996-1997	Blood	36	0.25->64	1.30	30.0	[5]
1999-2000	Blood	47	0.57-13	3.70	11.0	[5]
2002	Blood	50	0.25->64	2.40	10.7	Present study (disk-diffusion method)
2002	Non-blood	68	0.25->64	1.80	>64	Present study

[a] MIC_{50} and MIC_{90} are the minimum inhibitory concentrations at which 50% and 90% of the isolates tested were inhibited.

immunosuppression were risk factors for resistance of *Candida* [18]. However, false resistance determined by the disk-diffusion method has been reported [15]. Thus, fluconazole MICs should be further determined using the confirmatory broth dilution method NCCLS document (M27-A). However, the clinical significance of this phenomenon remains to be determined.

The species distribution in candidemia remained stable in the past 5 years at NTUH. Data from the National Nosocomial Infections Surveillance System in the USA revealed a significant decrease in the incidence of *C. albicans* bloodstream infection together with a significant increase in the incidence of *C. glabrata* bloodstream infection in the past 10 years, and that *C. glabrata* was the second most common spp. [19]. However, *C. tropicalis* fungemia remains the second most common spp. of *Candida* at NTUH and its proportion increased from 14% during 1981-1993 [1] to 22-23% during 1996-2002 [5]. When comparing data collected in different geographical regions of the world, the percentage of *C. tropicalis* (22%) was higher at NTUH than in the USA (10-12%) [20]. In Taiwan, there were also 2 studies of nosocomial candidemia in other hospitals which found a high percentage (20-21%) of *C. tropicalis*, with this organism ranking second among all spp. of *Candida* isolated [21,22]. But 2 other studies found a lower percentage (7-16%) [23,24]. Previous studies showed that patients with leukemia and neutropenia had a higher proportion of fungemia due to *C. tropicalis* [25,26]. Colonization of *C. tropicalis* also had higher predictive value of subsequent invasive candidiasis than other *Candida* spp. [8,27]. Our previous study showed that the MIC_{90} of *C. tropicalis* (13 μg/mL) was higher than that of *C. albicans* (4.6 μg/mL) and *C. parapsilosis* (1.42 μg/mL), and was comparable to that of *C. glabrata* (11 μg/mL) [5]. The present study further demonstrated a higher proportion of fluconazole resistance in non-blood isolates of *C. tropicalis* than in blood isolates. *C. tropicalis* resistance to fluconazole (48%) has been highlighted as a problem in the North West of England, with treatment failures leading to fatal outcomes [28]. One of the mechanisms of resistance in *C. tropicalis* may be drug efflux from the cell, and the acquisition of azole resistance develops very rapidly in an in vitro model [29]. Thus, the epidemiological significance of our data cannot be neglected.

The isolation of *Candida* from sputum, throat swab, urine, stool, or skin does not necessarily signify an invasive candidiasis. However, colonization with *Candida* was identified to be an independent predictor of candidemia [7,8]. *Candida* colonization of indwelling vascular devices, *Candida* urinary tract infection, and overgrowth of yeasts in the gastrointestinal tract can all lead to candidemia. Azole resistance is also frequently described in patients with AIDS and mucosal candidiasis, or oral candidiasis [16,30,31]. In the global evaluation of fluconazole susceptibility, the highest percentage of

fluconazole-resistant *C. albicans* strains was isolated from the upper respiratory tract, and the highest percentage of fluconazole-resistant *Candida* strains was isolated from the lower respiratory tract [10]. The present study showed that a higher percentage of fluconazole-resistant strains was obtained from the upper or lower respiratory tract. Increased use of fluconazole may lead to a shift toward intrinsically resistant *Candida* spp. such as *C. glabrata* and *C. krusei*, and the emergence of resistant strains of *C. albicans* [1,32,33].

The limitations of this study included relatively small numbers of non-blood isolates tested compared to the total number of clinical isolates. Programs for ongoing surveillance are needed to detect antifungal susceptibility and species-specific changes in the incidence of candidemia.

In conclusion, the disk-diffusion method for testing fluconazole susceptibility in *Candida* isolates is rapid and cost-effective. Evaluation of susceptibility to fluconazole in non-blood isolates is also crucial in the surveillance of antifungal resistance. Further population-based or sentinel surveillance of fluconazole susceptibility for evaluation of the effect of increasing fluconazole use may help to avoid loss of effectiveness of fluconazole as an antifungal agent.

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