Survey of amphotericin B susceptibility of *Candida* clinical isolates determined by Etest

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Background and Purpose: The minimal inhibitory concentrations (MICs) of amphotericin B (AmB) determined by the National Committee for Clinical Laboratory Standards (NCCLS; NCCLS document M27-A) broth dilution method are in a relatively narrow ranges and this may lead to underestimation of the AmB-resistant rate in clinical isolates. We evaluated in vitro susceptibility of clinical isolates of *Candida* spp. to AmB using Etest and determined the distribution of AmB MICs in different species.

Methods: We used the Etest (AB Biodisk, Solna, Sweden) to evaluate the MICs of *Candida* isolates randomly collected during 2001-2003 in a teaching hospital.

Results: Of the 572 isolates evaluated, *Candida albicans* (50.7%) was the most common species, followed by *Candida tropicalis* (23.9%), *Candida parapsilosis* (13.1%), *Candida glabrata* (9.4%), *Candida krusei* (1.9%), and *Candida guilliermondii* (0.9%). The majority of isolates were from blood (85%). The minimal concentrations of AmB required to inhibit 50%/90% of the isolates (MIC_{50}/MIC_{90}) were 0.19/0.38 μg/mL for *C. krusei*, 0.125/0.38 μg/mL for *C. glabrata*, 0.094/0.25 μg/mL for *C. tropicalis*, 0.032/0.19 μg/mL for *C. albicans*, 0.016/0.125 μg/mL for *C. parapsilosis*, and 0.023/0.032 μg/mL for *C. guilliermondii*. Only 1 blood isolate of *C. glabrata* was resistant to AmB (MIC ≥1 μg/mL) [0.17%]. 18.2% of isolates were less susceptible to AmB (MIC ≥0.19 μg/mL) with the highest rates for *C. krusei* (63.6%), followed by *C. glabrata* (37.0%), *C. tropicalis* (29.9%), *C. albicans* (11.0%), *C. parapsilosis* (5.3%), and *C. guilliermondii* (0%). More isolates collected from patients with hematologic malignancy were less susceptible to AmB than those collected from those with other diseases (30.5% vs 15.4%, *p*<0.05).

Conclusion: This study demonstrated that AmB resistance remains rare at this hospital in *Candida* clinical isolates despite increasing use of this agent during the past 4 decades.

Key words: Amphotericin B, *Candida*, fungal drug resistance, microbial sensitivity tests

Introduction

Nosocomial infections caused by yeasts have increased significantly in recent decades. *Candida* are the most common pathogens, being responsible for 80% of fungal infections [1-3]. *Candida* spp. have been the leading pathogens overall among nosocomial infections and nosocomial bloodstream infections since 1993 at a major

teaching hospital in Taiwan [4,5]. Amphotericin B (AmB), a polyene antibiotic, has remained active against most species of pathogenic fungi and has been the drug of choice for the treatment of most invasive fungal infections in the past 4 decades. Annual consumption of AmB has increased gradually during this period and was 0.5 g per 1000 patient-days at this hospital in 2000 [6]. Antifungal resistance has become an important issue for a variety of fungal infections [7], and AmB-resistant *Candida* isolates have been reported both in vivo and in vitro [8-11].

The National Committee for Clinical Laboratory Standards (NCCLS) broth dilution method for antifungal susceptibility testing of yeast [12] is useful for in vitro

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susceptibility testing of azoles. However, it is time-consuming, expensive, technically difficult to perform [10] and might be unable to detect some AmB resistant organisms [8,10] because the range of AmB minimal inhibitory concentrations (MICs) determined by this method is relatively narrow [10,13,14]. Thus, the true prevalence of AmB resistance might be underestimated when this technique is used [7,15]. On the other hand, the Etest, an alternative method for susceptibility testing of yeasts, may detect a broader range of AmB resistance in *Candida* isolates [10,16,17]. We evaluated in vitro susceptibility of clinical isolates of *Candida* spp. to AmB using Etest and determined the distribution of AmB MICs in different species.

Methods

Candida isolates

Clinical isolates of *Candida* spp. were collected randomly from the Mycology Laboratory of National Taiwan University Hospital from January 1, 2001 through December 31, 2003. The specimens from which these isolates were cultured included blood, urine, sputum, and otherwise sterile body fluid. Organisms were identified by germ tube analysis and morphology on cornmeal-Tween 90 agar [18] or, when necessary, by standard biochemical testing with the API 20C system (API BioMerieux Vitek Inc., Hazelwood, MO, USA). All of the isolates were kept at –70°C and were subcultured at least twice on Sabouraud dextrose agar (BBL Becton-Dickinson, Cockeysville, MD, USA) at 35°C prior to being tested. If the same *Candida* spp. was isolated from the same type of specimen during a 7-day period from the same patient, only the first isolate was included in this analysis.

In vitro susceptibility testing

The Etest (AB Biodisk, Solna, Sweden) was performed according to the manufacturer's instructions. The inoculum was prepared from 24-h cultures of *Candida* spp. Cell suspensions were prepared in sterile distilled water and adjusted to a concentration corresponding to a 0.5 McFarland standard for *Candida* spp. using a spectrophotometer set at 530 nm. The medium used was RPMI-1640 agar (with L-glutamine, without bicarbonate) [Sigma Chemical Co., St Louis, MO, USA], supplemented with glucose (2%) and buffered to pH 7.0 with 0.165 M 3-N-morpholinopropane-sulphonic acid (MOPS, Sigma). The plates were inoculated by dipping a sterile swab into the appropriate

cell suspension and streaking it across the entire surface of the agar in 3 directions. The plates were dried at room temperature for 15 min before the Etest strips were applied. The plates were incubated at 35°C and read at 24 h. The Etest MIC was read as the drug concentration at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip. Isolates with a MIC of ≥1 µg/mL [14] were considered to be resistant to AmB. Isolates with an MIC of ≥0.19 µg/mL were associated with a higher risk of therapeutic failure [19] and were considered to be less susceptible to AmB in this study.

Statistical methods

Before analyses, the low off-scale MICs were left unchanged. Relationships between categorical variables were analyzed with the chi-squared test, and between categorical and continuous variables with unpaired Student's *t*-test. All reported *p* values were 2-tailed and a *p* value of <0.05 was considered significant.

Results

Species distribution

A total of 632 *Candida* clinical isolates were collected and their in vitro susceptibility to AmB was determined. After deletion of the duplicate strains, 572 isolates collected from 470 patients were analyzed. Among the patients from whom these isolates were obtained, 444 (94.5%) had 1 isolate evaluated, 24 (5.1%) had 2 isolates and 2 (0.4%) had 3 different isolates. *Candida albicans* was the most common pathogen (50.7%) followed by *Candida tropicalis* (23.9%), *Candida parapsilosis* (13.1%), *Candida glabrata* (9.4%), *Candida krusei* (1.9%), and *Candida guilliermondii* (0.9%). Sources of isolates included blood (85%), airway (5.2%), urine (3.3%), wound (2.4%), and catheters (2.1%). Among the 26 patients with multiple isolates of *Candida* spp., different isolates were obtained from blood in 21 patients and from urine in 1 patient. Only 4 patients had different isolates from different sources, including blood and wound (n = 2), blood and airway (n = 1), as well as airway and urine (n = 1). Among the 145 isolates collected from patients with hematological malignancy, *C. albicans* was the most common pathogen (48 isolates), followed by *C. tropicalis* (34 isolates).

Amphotericin B susceptibility

The MICs of 572 isolates ranged from <0.002 µg/mL to 1.5 µg/mL (Table 1 and Fig. 1). The minimal

Table 1. In vitro activities of amphotericin B against different clinical isolates of *Candida* spp. collected during 2001-2003

Species	Minimal inhibitory concentration (µg/mL)		
	Range	MIC_{50}	MIC_{90}
Candida albicans (n = 290)	<0.002-1.5	0.032	0.19
Candida tropicalis (n = 137)	<0.002-0.38	0.094	0.25
Candida parapsilosis (n = 75)	<0.002-0.19	0.016	0.125
Candida glabrata (n = 54)	0.003-1.5	0.125	0.38
Candida krusei (n = 11)	0.008-0.5	0.19	0.38
Candida guilliermondii (n = 5)	0.012-0.032	0.023	0.032
Total	<0.002-1.5	0.047	0.19

Abbreviations: MIC_{50} = minimal concentration inhibiting 50% of isolates; MIC_{90} = minimal concentration inhibiting 90% of isolates

concentrations of AmB required to inhibit 50%/90% of the isolates (MIC_{50}/MIC_{90}) were 0.047/0.19 µg/mL. Among the different *Candida* spp., *C. krusei* and *C. glabrata* had higher MIC_{50} values (0.19 µg/mL and 0.125 µg/mL, respectively) than other *Candida* spp. (Table 1). The distributions of MICs of different *Candida* spp. varied considerably (Fig. 1).

*Candida* isolates from different sources had slightly different MICs of AmB (Table 2). The MIC_{50} of isolates from catheters and urine (0.125 µg/mL) were higher than those of isolates from other sources (0.032 to 0.094 µg/mL). The MIC_{90} of isolates from body fluid (0.5 µg/mL) were higher than those of isolates from

other sources (0.19 to 0.25 µg/mL). The MIC_{50} and MIC_{90} of blood isolates (0.032 µg/mL and 0.19 µg/mL, respectively) were the lowest among the different sources.

Using a breakpoint of ≥1 µg/mL, no AmB-resistant *C. krusei* isolate was identified. Only 1 *C. glabrata* blood isolate was resistant to AmB (1/572, 0.17%). This isolate was obtained from a patient with non-Hodgkin's lymphoma receiving steroid treatment who had previously received fluconazole for oral candidiasis 10 days before collection of blood cultures. This patient died 1 day after specimen collection during continued AmB treatment (0.7 mg/kg/day).

Overall, 18.2% of isolates were less susceptible to AmB (MIC ≥0.19 µg/mL) and this rate was highest among *C. krusei*, followed by *C. glabrata* and *C. tropicalis* (Table 3). More isolates collected from patients with hematologic malignancy were less susceptible to AmB than those collected from patients with other illnesses (30.5% vs 15.4%, *p*<0.05). Among the different *Candida* species, more isolates of *C. tropicalis* collected from patients with hematologic malignancy were less susceptible to AmB than those collected from patients with other illnesses (Table 3).

There was no correlation between AmB susceptibility and fluconazole susceptibility (determined either by Etest [data not shown] or disk diffusion method [6,20]) [R = 0.09 and 0.044, respectively].
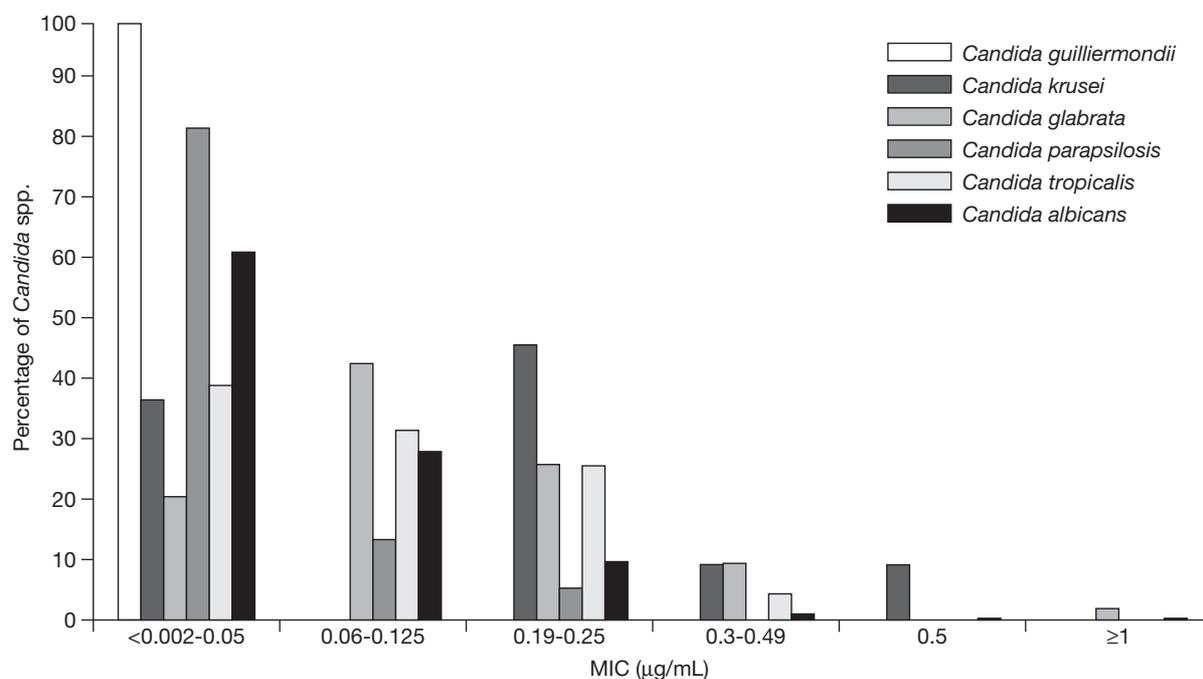
Fig. 1. The distribution of minimal inhibitory concentrations (MICs) of amphotericin B in different *Candida* spp.

Table 2. Amphotericin B susceptibilities of *Candida* spp. from different sources

Source/MIC (μg/mL)	Blood (n = 485)	Airway[a] (n = 30)	Urine (n = 19)	Wound (n = 14)	Catheters[b] (n = 12)	Body fluids[c] (n = 9)	Others[d] (n = 3)
Candida albicans	<0.002-0.5	0.006-0.25	0.012-0.25	0.064-0.19	0.125	0.003-0.125	0.023-0.094
MIC_{50}	0.032	0.032	0.064	0.125	0.125	0.032	0.023
MIC_{90}	0.19	0.19	0.25	0.19	0.125	0.125	0.094
Candida tropicalis	<0.002-0.38	0.012-0.25	0.032-0.25	0.016-0.38	0.032-0.19	0.016-0.25	0.125
MIC_{50}	0.064	0.125	0.064	0.094	0.125	0.016	0.125
MIC_{90}	0.25	0.25	0.25	0.38	0.19	0.25	0.125
Candida parapsilosis	<0.002-0.19	-	-	-	0.047	-	-
MIC_{50}	0.016	-	-	-	0.047	-	-
MIC_{90}	0.125	-	-	-	0.047	-	-
Candida glabrata	0.012-1.5	-	0.003-0.25	-	0.125	-	-
MIC_{50}	0.125	-	0.125	-	0.125	-	-
MIC_{90}	0.38	-	0.25	-	0.125	-	-
Candida krusei	0.008-0.38	0.19-0.25	-	0.041	0.19	0.5	-
MIC_{50}	0.016	0.25	-	0.041	0.19	0.5	-
MIC_{90}	0.38	0.25	-	0.041	0.19	0.5	-
Candida guilliermondii	0.012-0.032	-	-	-	-	-	-
MIC_{50}	0.023	-	-	-	-	-	-
MIC_{90}	0.032	-	-	-	-	-	-
Total	<0.002-1.5	0.006-0.25	0.003-0.25	0.016-0.38	0.032-0.19	0.003-0.5	0.023-0.125
MIC_{50}	0.032	0.094	0.125	0.094	0.125	0.032	0.094
MIC_{90}	0.19	0.25	0.25	0.25	0.19	0.5	0.125

Abbreviations: MIC_{50} = minimal concentration inhibiting 50% of isolates; MIC_{90} = minimal concentration inhibiting 90% of isolates
[a]Specimens collected from the airway included throat swab, sputum, and bronchial washing fluid.
[b]The catheters included central venous catheters, Port-A-Cath, and drainage tubes.
[c]Body fluid specimens included pleural effusion, ascites, and cerebrospinal fluid.
[d]This category comprised specimens collected from abscesses in solid organs.

Discussion

Previous studies [10,13,14,19,21] demonstrated that the NCCLS broth dilution method yielded a more narrow range of AmB MICs and had a limited ability to identify AmB-resistant *Candida* isolates. By contrast, this and other studies using Etest [10,13,19,21] found ranges of MICs which were broader than those determined by the NCCLS broth dilution method, thus allowing better discrimination of AmB MICs. The present study

found that AmB resistance (MIC ≥1 μg/mL) in *Candida* isolates remained low (0.17%), and the results were comparable to a recent multicenter study in Taiwan (0.5%) [22].

AmB resistance rates ranged from 0% to 3% in previous studies [15,23-25], with the highest rate (3%) reported from a tertiary care cancer center in the United States [15]. By contrast, no AmB resistance was found in a population-based active surveillance study in Spain during 2002-2003 [26]. Variation of AmB resistance in

Table 3. Percentages of *Candida* isolates less susceptible to amphotericin B (minimal inhibitory concentrations ≥0.19 μg/mL)

Species	No. of less susceptible isolates/no. of total isolates (%)		
	Total	Hematologic malignancy	Others
Candida albicans	32/290 (11.0)	8/48 (16.7)	24/242 (9.9)
Candida tropicalis[a]	41/137 (29.9)	19/34 (55.9)	22/103 (21.4)
Candida parapsilosis	4/75 (5.3)	0/13 (0)	4/62 (6.5)
Candida glabrata	20/54 (37.0)	1/4 (25.0)	19/50 (38.0)
Candida krusei	7/11 (63.6)	4/5 (80.0)	3/6 (50.0)
Candida guilliermondii	0/5 (0)	0/1 (0)	0/4 (0)
Total[a]	104/572 (18.2)	32/105 (30.5)	72/467 (15.4)

[a]p value of <0.05.

different series may result from differences in patient population [15], prior use of antifungal agents [27,28], distribution of *Candida* spp., and methods of in vitro susceptibility testing. AmB resistance in *Candida* spp. has been reported using different methodologies, different breakpoint values of in vitro AmB susceptibility and clinical outcome [14,15,19,23-26].

The attainable peak serum level with a daily dose of AmB at 0.6 mg/kg is about 1.4 µg/mL [29]. A multicenter observational study which used a breakpoint value of ≥1 µg/mL for AmB MICs determined using NCCLS broth dilution method demonstrated that in vitro susceptibility testing for AmB could predict microbiology failure and response to therapy in patients receiving AmB [14]. A similar trend was found in a subsequent retrospective study (2/3 [67%] vs 11/77 [14%], $p>0.05$) [15].

A study by Clancy and Nguyen found that patients whose isolates had AmB MICs ≥0.19 µg/mL determined at 24 h using Etest were at higher risk of therapeutic failure than those with AmB MICs <0.19 µg/mL (46% vs 17%, $p=0.005$) [19]. Comparison of antifungal susceptibility of different *Candida* spp. has established that *C. krusei* and *C. glabrata* are less susceptible to fluconazole than other *Candida* spp. [6,13,30-32]. In this study, the MIC_{50} and percentages of AmB less-susceptible isolates were higher among *C. krusei* and *C. glabrata* than other *Candida* spp. Data from a cancer center demonstrated that the AmB resistance rate was highest for *C. krusei* (24%), followed by *C. glabrata* (5%), while none of the other *Candida* spp. were found to be AmB resistant [15]. The differences in AmB susceptibility of different *Candida* spp. were similar to the pattern of AmB killing kinetics against different *Candida* spp. [33]. However, the clinical significance of these findings warrants further study.

In this study, only 1 AmB-resistant *C. glabrata* blood isolate was collected, from a patient with prior fluconazole use. However, 18.2% of isolates collected from patients with hematologic malignancy were less susceptible to AmB. Invasive fungal infections due to AmB-resistant *Candida* spp. have been increasingly reported [15,34-37] and associated with severely immunocompromised patients, previous polyene and cytotoxic chemotherapy exposure, as well as antifungal prophylaxis with azoles [27,28]. The mechanism of AmB resistance may be an alteration or a decrease in the amount of ergosterol in the cell membrane [27].

This study had several limitations. First, the clinical isolates were collected at a teaching hospital and thus cannot represent the overall prevalence of AmB resistance in Taiwan. Second, only a small number of non-blood isolates of non-*albicans Candida* spp. were evaluated. Third, as only 1 isolate in this study had an AmB MIC ≥1 µg/mL, we did not perform in vitro and in vivo correlation. Further programs for surveillance are needed to evaluate the correlation between outcome and in vitro susceptibility using different methodologies and breakpoints.

In conclusion, Etest is a useful method for AmB in vitro susceptibility testing for individual patients or large-scale population studies to detect AmB-resistant strains [10,16,17]. Despite its widespread clinical use for 4 decades and increasing annual consumption during the past decade at this teaching hospital in Taiwan [6], resistance to AmB remains rare. However, a significant proportion of *C. krusei* and *C. glabrata* isolates had reduced susceptibility to AmB. As AmB remains an important antifungal agent for the treatment of invasive candidiasis, the importance of rational use of AmB as well as fluconazole with adequate doses cannot be over-emphasized.

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