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Brief communication (Original)

Correlation between broth microdilution, E-test and disk diffusion methods for testing antifungal susceptibility of *Candida* species isolated from Thai blood samples

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Background: Broth microdilution (BMD) is a standard assay for susceptibility of *Candida* to antifungals, but complexity limits its routine application. E-test (ET) and disk diffusion (DD) assays are attractive alternatives because of their simplicity and reproducibility.
Objectives: To determine the correlation between BMD, and ET and DD assay results for *Candida* isolates.
Methods: We tested 63 *Candida* isolates for their susceptibility to fluconazole and voriconazole using BMD, ET, and DD, and recorded minimum inhibitory concentrations (MIC) and inhibitory zone diameters (ZD). Spearman correlations were determined and the Clinical and Laboratory Standards Institute recommendations were used to assess major and minor errors in test results.
Results: The isolates included 32 (51%) *C. albicans*, 14 (22%) *C. tropicalis*, 12 (19%) *C. parapsilosis*, 4 (6%) *C. glabrata*, and 1 (2%) *C. guilliermondii*. The BMD-MIC and ET-MIC had good correlation for fluconazole ($r = 0.94$; $P < 0.001$) and voriconazole ($r = 0.95$; $P < 0.001$). The BMD-MIC for both antifungals were significantly correlated with the ZD of the DD assays ($r = -0.47$; $P < 0.001$; $r = -0.75$; $P < 0.001$, respectively). Agreement between the BMD and the ET and DD results exceeded 90%. No major errors were identified in any comparisons.
Conclusions: *C. albicans* were predominant among the isolates and were susceptible to fluconazole and voriconazole. The BMD results were well correlated with ET and DD assay results, and therefore ET and DD assays can be recommended as initial screening tools in resource-limited hospitals because of their relatively low cost.

Keywords: Broth microdilution, candidiasis, disk diffusion, E-test

Candidiasis is the most common fungal infection of blood [1, 2]. Candidemia in hospitalized patients is associated with a higher attributable mortality, ranging from 5%–71%, a significantly increased cost of healthcare, and an increased length of hospital stay [3, 4]. Despite advances in healthcare, invasive candidiasis-associated mortality has remained stable for the past 2 decades, although a decreased mortality rate has been observed for other fungal infections e.g. invasive aspergillosis [5]. *Candida albicans* is the predominant *Candida* species accounting for over half of all cases of candidemia, but the incidence of non-*albicans Candida* species has been increasing in the last decade throughout the world. In particular, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*, now comprise infections in one-fourth of all patients with candidemia [6].

Fluconazole remains the initial choice of therapy for candidemia as recommended by the Infectious Disease Society of America [7]. However, fluconazole-resistant strains, particularly of non-*Candida albicans* species, have been increasing in

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prevalence [6]. The continuously changing patterns of *Candida* species and the rising incidence of antifungal-resistance in less-common species, which vary between different geographic areas, means that performing antifungal susceptability testing is crucial in guiding treatment strategies [8].

The broth microdilution (BMD) method is the standard approved reference method for *Candida* susceptibility testing according to Clinical and Laboratory Standard Institute (CLSI) guidelines [9]. However, its complexity, requiring experienced laboratory technicians, limit its availability for routine laboratory testing in resource-limited hospitals. To overcome these limitations, agar-based methods including the E-test (ET) and disk diffusion (DD) methods have become attractive alternatives because of their simplicity, reproducibility, and lack of the need for specialized equipment. Other studies have suggested that ET and DD show comparable results to the standard method of BMD [10-12]. The DD method in particular, being widely commercially available and inexpensive (its cost is ten-fold lower than that of ET), has become the most acceptable alternative for community settings, although one study has reported low correlation with BMD for antifungal-resistant isolates [12].

The aim of the present study was to evaluate the performance of DD and ET assays compared with the standard BMD method, and to validate the accuracy of these agar-based methods for initial routine laboratory screening for antifungal susceptibilities of local strains in Thailand.

Materials and methods

Study design and data collection

A total of 63 isolates of *Candida* species were obtained routinely from the blood of patients with candidemia in King Chulalongkorn Memorial Hospital, a 1500-bed single tertiary-care center in Thailand, over a period of 3 years (January 2007 to December 2010), and identified in the Department of Microbiology. Isolates from same patient that were obtained within one week were considered as duplicates and excluded. BD BACTEC Myco/F Lytic culture medium (Becton, Dickinson and Company, Sparks, MD, USA) was used for culture of all *Candida* isolates included in this study. Coded (linked anonymized) isolates that could not be directly identified with patients were stored at –80°C in Sabouraud dextrose broth with beads (BD Diagnostics, Franklin lakes, NJ, USA). These isolates were then subcultured at least twice on Sabouraud dextrose agar at 35°C to confirm viability and purity before susceptibility testing in the present study. The present study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB 168/54 and 315/58, COA Nos. 258/2011 and 569/2015).

Species identification

All isolates were cultured on chromogenic agar (CHROMagar, Paris, France) for presumptive identification and to exclude contaminants. Species identity of isolates was confirmed using an API 20C Aux yeast identification system (bioM rieux, Montalieu, France) [13]. Additional tests to confirm the identity of species included morphological examinations such as the germ tube test, chlamydospore production test, and colony morphology observation.

Broth microdilution

All *Candida* isolates were tested for their in vitro susceptibility to voriconazole (Pfizer, Groton, CT, USA) and fluconazole (Pfizer) by BMD according to the approved standard reference method described in CLSI document M27-A3 [9].

Three laboratory technicians determined MICs independently at 24 h and 48 h after incubation at 35°C. Because MIC values at 24 h and 48 h were found to be comparable (data not shown) we used only the 48 h readings for this analysis. The MIC values were based on consensus of at least 2 of the 3 technicians, otherwise the susceptibility test was repeated until a consensus was established. Interpretation of susceptibility was performed by applying the clinical breakpoints (CBPs) defined by CLSI document M27-A3 [9].

E-test

An inoculum of $1–5 \times 10^6$ cells/ml was used and applied to the agar plates (RPMI 1640 with 2% glucose) according to the manufacturer's instructions. E-test strips (bioM rieux) were then applied. MICs were determined after 24 h and 48 h of incubation and interpreted according to CLSI document M27-A3 [9].

Disk diffusion

An inoculum of $1–5 \times 10^6$ cells/ml was used and disks applied to Mueller–Hinton agar with 2% glucose according to the manufacturer's instructions (Bio-Rad

Life Science, Marnes-la-Coquette, France). The plates were incubated at 35°C and read at 24 h and 48 h. Inhibitory zone diameters were interpreted according to CLSI document M44-A at the transitional point where growth abruptly decreased [14].

Quality control

All methods were validated using quality control strains of *C. albicans* ATCC 90028, *C. tropicalis* ATCC750, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258.

Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 21.0 for Mac (IBM Corp, Armonk, NY, USA). Descriptive statistics were used to present the data. The MIC values were reported as a range. $P < 0.05$ was considered significant. A Spearman correlation was used to determine agreement between BMD-MIC and ET-MIC values, and BMD-MIC values and DD assay diameters.

For the three-way comparison of BMD, ET, and DD, correlations were determined as recommended by the CLSI: (i) the definition of the occurrence of a very major error (VME) is when one strain is susceptible by the test methods (DD or ET), but is resistant by the BMD reference method; (ii) major errors (MEs) are defined as occurring when an isolate is found to be resistant by the test methods (ET or DD), but is susceptible by the BMD reference method; (iii) a minor error (mE) is indicated when an isolate is susceptible and dose dependent by one method, but susceptible or resistant by other methods. The total percentage agreement was calculated by the proportion of all test isolates that are in agreement, minus any errors, divided by the total number of test isolates.

Results

Species distribution of isolates from candidemia

A total of 63 *Candida* isolates were tested including 32 (51%) *C. albicans*, 14 (22%) *C. tropicalis*, 12 (19%) *C. parapsilosis*, 4 (6%) *C. glabrata*, and 1 (2%) *C. guilliermondii.*

C. albicans isolates were highly susceptible to fluconazole (100%) and voriconazole (91%). No resistant strain of *C. albicans* was detected and only 3 strains were identified as susceptible–dose dependent (S-DD) against voriconazole.

C. tropicalis and *C. parapsilosis* were the majority (41%) of non-*C. albicans Candida* isolates. These species were also highly susceptible to fluconazole (93%–100%) and voriconazole (79%–100%). Only one resistant strain (*C. parapsilosis*) was found against fluconazole and one resistant strain (*C. tropicalis*) was found against voriconazole. Antifungal susceptibilities of each *Candida* species are detailed in **Table 1**.

Table 1. Antifungal susceptibilities of 63 *Candida* isolates.

Antifungal agent	*Candida*	MIC range (μg/mL)	No. (%) of isolates indicated susceptibility category by CBPs		
			S	S-DD	R
Fluconazole	C. *albicans* (32)	0.06–2	32 (100)	0	0
	C. tropicalis (14)	0.06–64	13 (93)	0	1 (7)
	C. parapsilosis (12)	0.125–4	12 (100)	0	0
	C. glabrata (4)	0.375–4	4 (100)	0	0
	C. guilliermondii (1)	1–1	1 (100)	0	0
Voriconazole	C. *albicans* (32)	0.06–2	29 (91)	3 (9)	0
	C. tropicalis (14)	0.03–4	11 (79)	2 (14)	1 (7)
	C. parapsilosis (12)	0.06–1	12 (100)	0	0
	C. glabrata (4)	1–4	2 (50)	1 (25)	1 (25)
	C. guilliermondii (1)	0.5–0.5	1 (100)	0	0

MIC, minimum inhibitory concentration; CBPs, clinical breakpoints defined by CLSI document M27-A3 [9]: S, susceptible; S-DD, susceptible–dose dependent; R, resistant.

Comparison of BMD, ET, and DD

The BMD-MIC values showed good correlation with ET-MIC values for fluconazole and voriconazole (r = 0.95; P < 0.001 and r = 0.94; P < 0.001 respectively). The BMD-MIC values were significantly correlated with inhibitory zone diameters observed for the DD assays for fluconazole and voriconazole (r = –0.47; P < 0.001 and r = –0.75; P < 0.001, respectively).

The total percentage agreement of the ET and DD determinations, compared with the standard reference BMD-MIC values for the two antifungal agents, exceeded 90% as summarized in **Table 2**.

The percentage agreement for the ET assays ranged between 92% to 98% and for DD between 92% to 98%. No VMEs were detected and only one ME was identified for a C. *tropicalis* isolate tested by DD with fluconazole. The mE detection rates ranged between 2% to 8% for ET determinations and 2% to 6% for DD determinations.

Discussion

Determining the species distribution and susceptibility profiles of the yeast isolated from cases of invasive candidiasis is essential to guide physicians in the choice of antifungal agents in any empirical treatment, because these susceptibility profiles vary geographically and not all hospitals perform antifungal susceptibility tests routinely. Therefore, this information is crucial and currently lacking for Thailand and other places in South-east Asia. The last published data from Thailand were in 2002 [15]. The present study provides an incidence and susceptibility profile for *Candida* isolated from blood in Thailand.

The predominant species was *C. albicans* (51%) and this was similar to that of previous studies in Southeast Asia and the ARTEMIS global surveillance study [6, 15-17]. The most common non-*Candida albicans* species in Thailand was *C. tropicalis* (22%) followed by *C. parapsilosis* (19%). These findings are consistent with data from other studies in Southeast Asia and China demonstrating that these two species are the predominant non-*Candida albicans* species. However in China and Malaysia, data have demonstrated a higher prevalence of *C. parapsilosis* than *C. tropicalis* [15-18]. Collectively, these results are in contrast with those from the USA and the ARTEMIS global surveillance study where C. *glabrata* was the most common non-*Candida albicans* species [6, 19]. These variations emphasize the importance of determining local epidemiologic patterns.

BMD as the standard antifungal testing method recommended by the CLSI can be difficult to perform in routine laboratory practice because of its complexity, the need of experienced laboratory technicians, and labor-intensive nature. By contrast, agar-based testing methods, (in particular DD, which has a ten-fold lower cost than ET) are commercially available, less expensive than BMD, easy to perform, and widely available, making them feasible as cost-effective screening methods. Our study demonstrated that BMD-MIC values showed good correlations with both ET-MIC and DD-MIC values. For the ET results, no VME was observed and the ME rates (92%–98%) were within the CLSI limits recommended in their document M-27A3 [9]. The DD test results also demonstrated an excellent performance (92%–98%) and no VME was observed. Only 1 ME was found

Table 2. Errors with the E-test and disk diffusion methods compared with broth microdilutions for fluconazole and voriconazole

Method	Category	Fluconazole by BMD			No. of strains			Total agreement (%)	Voriconazole by BMD			No. of strains			Total agreement (%)
		S	I	R	VME	ME	mE		S	I	R	VME	ME	mE	
ET	S	61	0	0	0	0	1	98	54	2	0	0	0	5	92
	I	1	0	0					1	4	2				
	R	0	0	1					0	0	0				
DD	S	57	0	0	0	1	4	92	55	1	0	0	0	1	98
	I	4	0	0					0	5	0				
	R	1	0	1					0	0	2				

BMD, broth microdilution; ET, E-test; DD, disk diffusion; VME, very major error; ME, major error; mE, minor error; S, susceptible; I, susceptible–dose dependent; R, resistant.

for fluconazole against C. *tropicalis* in DD. *C. tropicalis* MEs were also observed in a previous report [11]; therefore, laboratories should be aware of this possibility when performing DD with *C. tropicalis* species, and a standard BMD test may be warranted to confirm the antifungal susceptibility of *C. tropicalis*.

We found that the agar-based ET and DD assays are reliable alternatives to the standard reference BMD method for isolates that are susceptible to fluconazole and voriconazole, because they showed >90% agreement with the BMD method. The present study validates these agar-based methods for use as initial screening tools in Thailand [11, 12, 20]. A limitation of our study is that we only selected *Candida* isolates from blood; further study should include other sterile sites including cerebrospinal fluid, intravascular catheter samples or other sterile tissue samples for an improved validation of the agar-based methods.

In summary, DD is a valid alternative method to BMD for testing *Candida* susceptibility to fluconazole and voriconazole and can be performed as a cost-effective screening test in community settings in Thailand as a result of its economy and commercial availability.

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Conflict of interest statement

Sansanee Lerdlitruangsin is an employee of MSD (Merck Sharp & Dohme) Thailand Ltd, a subsidiary of Merck & Co, Whitehouse Station, NJ, USA. No other authors have any conflict of interest to declare.

Authors' contributions

AC, NW, DA, AT, and SL contributed to the acquisition, analysis, and interpretation of the data and drafted the article. VJ, WD, KK and SJ also drafted the article and critically revised it for important intellectual content. All authors read and approved the final manuscript.

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