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Disk Diffusion Susceptibility Testing for the Rapid Detection of Fluconazole Resistance in *Candida* Isolates

Suhak Jeon, M.D.[1], Jong Hee Shin, M.D., Ph.D.[1], Ha Jin Lim, M.D.[1], Min Ji Choi, Ph.D.[1], Seung A Byun, M.S.[1], Dain Lee, M.S.[1], Seung Yeob Lee, M.D.[2], Eun Jeong Won, M.D.[3], Soo Hyun Kim, M.D.[1,4], and Myung Geun Shin, M.D.[1]

[1]Department of Laboratory Medicine, Chonnam National University Medical School and Chonnam National University Hospital, Gwangju, Korea; [2]Department of Laboratory Medicine, Jeonbuk National University Medical School and Jeonbuk National University Hospital, Jeonju, Korea; Departments of [3]Parasitology and Tropical Medicine and [4]Microbiology and Laboratory Medicine, Chonnam National University Medical School, Hwasun, Korea

Background: Given the increased fluconazole resistance (FR) among *Candida* isolates, we assessed the suitability of disk diffusion susceptibility testing (DDT) for the early detection of FR using well-characterized *Candida* isolates.

Methods: In total, 188 *Candida* isolates, including 66 *C. albicans* (seven Erg11 mutants), 69 *C. glabrata* (33 Pdr1 mutants), 29 *C. parapsilosis* (15 Erg11 mutants), and 24 *C. tropicalis* (eight Erg11 mutants) isolates, were tested in this study. FR was assessed using DDT according to the standard CLSI M44-ED3 method, except that two cell suspensions, McFarland 0.5 (standard inoculum) and 2.5 (large inoculum), were used, and the inhibition zones were read at 2-hour intervals from 10 hours to 24 hours.

Results: DDT results for the standard inoculum were readable after 14 hours (*C. albicans*, *C. glabrata*, and *C. tropicalis*) and 20 hours (*C. parapsilosis*) for >95% of the isolates, whereas the results for the large inoculum were readable after 12 hours (*C. glabrata* and *C. tropicalis*), 14 hours (*C. albicans*), and 16 hours (*C. parapsilosis*) for >95% of the isolates. Compared with the results produced using the CLSI M27-ED4 broth microdilution method, the first readable results from the DDT method for each isolate exhibited an agreement of 97.0%, 98.6%, 72.4%, and 91.7% for the standard inoculum and 100%, 98.6%, 96.6%, and 95.8% for the large inoculum for *C. albicans, C. glabrata, C. parapsilosis*, and *C. tropicalis*, respectively.

Conclusions: DDT using large inoculum may detect FR rapidly and reliably in the four most common *Candida* species.

Key Words: *Candida*, Fluconazole, Resistance, Disk diffusion, CLSI, Antifungal susceptibility testing

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Corresponding author:
Jong Hee Shin, M.D., Ph.D.
Department of Laboratory Medicine, Chonnam National University Medical School, 42 Jebong-ro, Dong-gu, Gwangju 61469, Korea
Tel: +82-62-220-5342
Fax +82-62-224-2518
E-mail: shinjh@chonnam.ac.kr

INTRODUCTION

Candida is an important fungal pathogen that cause invasive candidiasis, leading to significant morbidity and mortality worldwide [1, 2]. Most cases of invasive candidiasis are attributable to *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* [3]. Fluconazole is among the most widely prescribed antifungal agents and is an important first-line treatment for invasive candidiasis owing to its potent efficacy, low cost, and broad-spectrum activity against various *Candida* species [4, 5]. However,

the widespread use of azole antifungals has resulted in the emergence of *Candida* isolates with acquired fluconazole resistance (FR), resulting in recent revisions of the clinical breakpoints (CBPs) because of the wide array of molecular mechanisms underlying resistance [3, 6, 7]. Therefore, fluconazole susceptibility testing is recommended for all bloodstream infections and clinically relevant *Candida* isolates, as it is useful in optimizing the treatment of invasive candidiasis [5].

Disk diffusion testing (DDT), one of the oldest approaches to susceptibility testing, is still widely used in clinical laboratories because of its low cost and technical simplicity [8]. DDT has recently been used for the direct testing of bacterial or fungal susceptibility in positive blood cultures [9–11]. Methods for shortening the incubation times for DDT have recently been reported for several common bacterial pathogens, enabling faster reporting of antimicrobial susceptibility test results from positive blood cultures [9, 12]. However, methods for shortening the incubation times for DDT for the detection of FR *Candida* isolates are less reported. Further, fluconazole susceptibility testing of *Candida* isolates using the DDT method has not been thoroughly evaluated, and previous studies using comparison with the CLSI M27 broth microdilution (BMD) reference method relied on tentative interpretive criteria that were applied to all *Candida* species [8, 13, 14].

We evaluated the performance of the DDT for the early detection of FR in four common *Candida* species using a panel of well-characterized *Candida* isolates from a Korean collection composed of FR isolates with various molecularly defined azole resistance mechanisms as well as a panel of wild-type isolates without detectable azole resistance mechanisms. Categorical fluconazole susceptibility results obtained using the DDT were compared with those obtained using the CLSI M27-ED4 BMD reference method, using revised species-specific CBPs, as defined in the CLSI M60-ED2 document [15, 16]. To our knowledge, ours is the first study to evaluate the performance of DDT for the early detection of FR among *Candida* isolates using a panel of *Candida* isolates with known and unknown resistance mechanisms.

MATERIALS AND METHODS

Candida isolates and azole resistance mechanisms

In total, 188 clinical isolates (186 from blood, one from urine, and one from pus) of four common *Candida* species from a Korean collection were tested. The collection included 66 *C. albicans*, 69 *C. glabrata*, 29 *C. parapsilosis*, and 24 *C. tropicalis* isolates that were submitted to Chonnam National University Hospital, Gwangju, Korea, from several Korean hospitals during a nationwide surveillance (2003–2019). All isolates were stored at –70°C in trypticase soy broth supplemented with 15% glycerol until testing and were passaged twice on potato dextrose agar at 35°C prior to testing in 2019. The isolates were identified to the species level using biochemical tests, matrix-assisted laser-desorption ionization-time of flight mass spectrometry, and/or molecular methods [17–19]. FR of all isolates was determined using the CLSI M27 BMD method [15]. For all *C. albicans* and *C. parapsilosis* isolates, *ERG11* and three transcription factor genes, i.e., *TAC1, MRR1*, and *UPC2*, were sequenced [7, 20]. For all *C. glabrata* isolates, *PDR1* was sequenced [21]. For all *C. tropicalis* isolates, *CDR1, MDR1*, and *ERG11* expression was quantified, and *ERG11* and *UPC2* were sequenced [22]. The full study panel consisted of *C. albicans* (59 wild-type fluconazole-susceptible [FS] isolates and seven FR isolates harboring the K143R or G464S variant in Erg11p), *C. glabrata* (36 wild-type FS dose-dependent [F-SDD] isolates and 33 FR isolates harboring a Pdr1 variant), *C. parapsilosis* (14 wild-type FS isolates and 15 FR isolates harboring the Y132F variant in Erg11p), and *C. tropicalis* (16 wild-type FS isolates, seven FR isolates overexpressing *ERG11*, and one FR isolate harboring the Y132F variant in Erg11p) isolates (Table 1). This study was approved by the Institutional Review Board of Chonnam National University Hospital, Gwangju, Korea (IRB CNUH-2018-283) which waived the requirement for informed consent.

Antifungal susceptibility testing

For all *Candida* isolates, the fluconazole minimal inhibitory concentration (MIC) was re-determined by the CLSI M27 BMD method after 24 hours of incubation [15]. Fluconazole MICs were categorized using species-specific CBPs provided in CLSI M60-ED2 document, as follows: MIC ≤2 μg/mL (FS), 4 μg/mL (F-SDD), and ≥8 μg/mL (FR) for *C. albicans*, *C. parapsilosis*, and *C. tropicalis*, and MIC ≤32 μg/mL (F-SDD) and ≥64 μg/mL (FR) for *C. glabrata* [16]. DDT was performed according to the CLSI M44-ED3 method using a 25-μg fluconazole disk (Oxoid, Basingstoke, UK) placed on Mueller-Hinton agar supplemented with 2% dextrose and 0.5 μg/mL methylene blue [23]. Inoculation was performed using cell suspensions of McFarland 0.5 (standard inoculum) and 2.5 (large inoculum), and results were read at 2-hour intervals from 10 hours to 24 hours. Average cell numbers (ranges) of each *Candida* species were $2.3–6.1\times10^6$ ($1.5–8.3\times10^6$) colony-forming units (CFU)/mL for the standard inoculum and $1.3–3.1\times10^7$ ($0.9–3.9\times10^7$) CFU/mL for the large in-

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Table 1. Categorical agreement between fluconazole susceptibility results determined by the BMD and DDT for 188 *Candida* isolates with known and unknown molecular mechanisms

Species	Fluconazole category by CLSI M27*	Defined resistance mechanism	N of isolates	N of isolates with DDT result for fluconazole†			% CA	N (%) of isolates with discrepancies†		
				FR	F-SDD	FS		VME	ME	Minor
C. albicans	FR	*ERG11*, K143R or G464S variant	7	5	0	2				
	FS	No variants (*ERG11*, *TAC1*, *MRR1*, and *UPC2*) defined	59	0	0	59				
	Total		66	5	0	61	97.0	2 (3.0)	0 (0.0)	0 (0.0)
C. glabrata	FR	*PDR1* variants	33	33	0	0				
	F-SDD	No *PDR1* variants	36	1	35	0				
	Total		69	34	35	0	98.6	0 (0.0)	0 (0.0)	1 (1.4)
C. parapsilosis	FR	*ERG11*, Y132F variant	15	12	2	1				
	FS	No variants (*ERG11*, *TAC1*, *MRR1*, and *UPC2*) defined	14	0	0	14				
	Total		29	12	2	15	89.7	1 (3.4)	0 (0.0)	2 (6.9)
C. tropicalis	FR	*ERG11* overexpression or Y132F variant	8	7	1	0				
	FS	No variants (*ERG11* and *UPC2*) defined	16	0	0	16				
	Total		24	7	1	16	95.8	0 (0.0)	0 (0.0)	1 (4.2)
Total	FR	Presence of defined azole resistance mechanisms	63	57	3	3				
	FS/F-SDD	No azole variant defined	125	1	35	89				
	Total		188	58	38	92	96.3	3 (1.6)	0 (0.0)	4 (2.1)

*Fluconazole susceptibility testing was performed according to the CLSI M27 BMD-ED4 method [15]. Categorical results were determined using the CLSI M60-ED2 method [16]. †Fluconazole susceptibility testing was performed according to the CLSI M44-ED3 method [23]. Categorical results were determined using the CLSI M60-ED2 method [16].
Abbreviations: BMD, broth microdilution; DDT, disk diffusion testing; CA, categorical agreement; FR, fluconazole resistant; FS, fluconazole susceptible; F-SDD, fluconazole-susceptible dose-dependent; ME, major error; Minor, minor error; VME, very major error.

oculum (Table 2).

The diameter of the growth inhibition zone was measured across the area in which growth was prominently reduced. The interpretive criteria for DDT are as follows: an inhibition zone diameter ≥17 mm indicates FS, a diameter of 14–16 mm indicates F-SDD, and a diameter ≤13 mm indicates FR for *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. For *C. glabrata*, an inhibition zone diameter ≥15 mm indicates F-SDD, and a diameter ≤14 mm indicates FR [16]. In each test, two reference isolates (*C. parapsilosis* American Type Culture Collection (ATCC) 22019 and *C. krusei* ATCC 6258) were included as controls.

Data and statistical analyses

Categorical agreement (CA) was determined as the percentage of isolates classified into the same FS or FR categories using both DDT and the CLSI M27 BMD method. Errors were classified as a very major error (VME) when an isolate classified as FR by the reference BMD method was categorized as FS by DDT, a

Table 2. Number of yeast cells/mL of the 0.5 and 2.5 McFarland suspensions of four common *Candida* species

Species	Inoculum*	Number of cells ($\times 10^6$ CFU/mL)†	
		Mean ± SD	Range
C. albicans	Standard	5.2 ± 0.9	3.8–6.9
	Large	26.5 ± 4.3	20.3–34.7
C. glabrata	Standard	6.1 ± 1.1	4.3–8.3
	Large	31.2 ± 4.8	23.3–39.0
C. parapsilosis	Standard	3.4 ± 0.6	2.5–4.2
	Large	17.0 ± 2.5	13.1–20.7
C. tropicalis	Standard	2.3 ± 1.0	1.5–4.7
	Large	13.3 ± 5.6	8.5–26.8
Total	Standard	4.3 ± 1.8	1.5–8.3
	Large	22.0 ± 8.4	8.5–39.0

*Colony counting was performed with the use of two cell suspensions (McFarland 0.5, standard inoculum; and 2.5, large inoculum). †Quantification was performed using a Neubauer chamber. Mean values of 10 isolates of each species are presented.

Table 3. Categorical agreement between fluconazole susceptibility results determined by DDT using two inocula and the BMD method after each incubation time for the readable *Candida* isolates*

Species (N tested)	Incubation time (hr)	DDT using a standard inoculum (McFarland 0.5)					DDT using a large inoculum (McFarland 2.5)				
		Readable N (%)	% CA	N (%) of isolates with discrepancies			Readable N (%)	% CA	N (%) of isolates with discrepancies		
				VME	ME	Minor			VME	ME	Minor
C. albicans (66)	10	5 (7.6)	100.0	0	0	0	26 (39.4)†	100.0	0	0	0
	12	38 (57.6)	100.0	0	0	0	55 (83.3)†	96.4	1 (1.8)	0	1 (1.8)
	14	63 (95.5)	96.8	2 (3.2)	0	0	63 (95.5)†	96.8	1 (1.6)	0	1 (1.6)
	16	63 (95.5)	95.2	2 (3.2)	0	1 (1.6)	66 (100.0)	95.5	1 (1.5)	0	2 (3.0)
	18	63 (95.5)	95.2	2 (3.2)	0	1 (1.6)	66 (100.0)	95.5	2 (3.0)	0	1 (1.5)
	20	64 (97.0)	96.9	2 (3.1)	0	0	66 (100.0)	95.5	1 (1.5)	0	2 (3.0)
	22	66 (100.0)	97.0	2 (3.0)	0	0	66 (100.0)	97.0	1 (1.5)	0	1 (1.5)
	24	66 (100.0)	97.0	2 (3.0)	0	0	66 (100.0)	97.0	1 (1.5)	0	1 (1.5)
C. glabrata (69)	10	46 (66.7)	100.0	0	0	0	60 (87.0)†	100.0	0	0	0
	12	60 (87.0)	100.0	0	0	0	68 (98.6)†	98.5	0	0	1 (1.5)
	14	67 (97.1)	98.5	0	0	1 (1.5)	69 (100.0)	98.6	0	0	1 (1.4)
	16	69 (100.0)	98.6	0	0	1 (1.4)	69 (100.0)	98.6	0	0	1 (1.4)
	18	69 (100.0)	98.6	0	0	1 (1.4)	69 (100.0)	98.6	0	0	1 (1.4)
	20	69 (100.0)	98.6	0	0	1 (1.4)	69 (100.0)	98.6	0	0	1 (1.4)
	22	69 (100.0)	98.6	0	0	1 (1.4)	69 (100.0)	97.1	0	0	2 (2.9)
	24	69 (100.0)	98.6	0	0	1 (1.4)	69 (100.0)	94.2	0	0	4 (5.8)
C. parapsilosis (29)	10	0 (0.0)	-	0	0	0	2 (6.9)	100.0	0	0	0
	12	1 (3.4)	100.0	0	0	0	24 (82.8)†	95.8	0	0	1 (4.2)
	14	19 (65.5)	68.4	1 (5.3)	0	5 (26.3)	26 (89.7)†	92.3†	0	0	2 (7.7)
	16	22 (75.9)	90.9	0	0	2 (9.1)	29 (100.0)†	93.1	0	0	2 (6.9)
	18	25 (86.2)	92.0	0	0	2 (8.0)	29 (100.0)†	93.1	0	0	2 (6.9)
	20	29 (100.0)	89.7	2 (6.9)	0	1 (3.4)	29 (100.0)	93.1	0	0	2 (6.9)
	22	29 (100.0)	89.7	2 (6.9)	0	1 (3.4)	29 (100.0)	93.1	0	0	2 (6.9)
	24	29 (100.0)	89.7	1 (3.4)	0	2 (6.9)	29 (100.0)	96.6	0	0	1 (3.4)
C. tropicalis (24)	10	15 (62.5)	93.3	0	0	1 (6.7)	22 (91.7)†	95.5	1 (4.5)	0	0
	12	21 (87.5)	90.5	1 (4.8)	0	1 (4.8)	24 (100.0)	87.5	1 (4.2)	0	2 (8.3)
	14	24 (100.0)	91.7	1 (4.2)	0	1 (4.2)	24 (100.0)	91.7	1 (4.2)	0	1 (4.2)
	16	24 (100.0)	91.7	1 (4.2)	0	1 (4.2)	24 (100.0)	95.8	1 (4.2)	0	0
	18	24 (100.0)	95.8	1 (4.2)	0	0	24 (100.0)	95.8	1 (4.2)	0	0
	20	24 (100.0)	95.8	0	0	1 (4.2)	24 (100.0)	95.8	1 (4.2)	0	0
	22	24 (100.0)	95.8	0	0	1 (4.2)	24 (100.0)	95.8	0	0	1 (4.2)
	24	24 (100.0)	95.8	0	0	1 (4.2)	24 (100.0)	95.8	0	0	1 (4.2)
Total (188)	10	66 (35.1)	98.5	0	0	1 (1.5)	110 (58.5)†	99.1	1 (0.9)	0	0
	12	120 (63.8)	98.3	1 (0.8)	0	1 (0.8)	171 (91.0)†	95.9	2 (1.2)	0	5 (2.9)
	14	173 (92.0)	93.6	4 (2.3)	0	7 (4.0)	182 (96.8)†	96.2	2 (1.1)	0	5 (2.7)
	16	178 (94.7)	95.5	3 (1.7)	0	5 (2.8)	188 (100.0)†	96.3	2 (1.1)	0	5 (2.7)
	18	181 (96.3)	96.1	3 (1.7)	0	4 (2.2)	188 (100.0)†	96.3	3 (1.6)	0	4 (2.1)
	20	186 (98.9)	96.2	4 (2.2)	0	3 (1.6)	188 (100.0)	96.3	2 (1.1)	0	5 (2.7)
	22	188 (100.0)	96.3	4 (2.1)	0	3 (1.6)	188 (100.0)	96.3	1 (0.5)	0	6 (3.2)
	24	188 (100.0)	96.3	3 (1.6)	0	4 (2.1)	188 (100.0)	95.7	1 (0.5)	0	7 (3.7)

*BMD method was conducted strictly according to CLSI M27-ED4 document (24-hour incubation), but DDT was modified with the use of two cell suspensions (McFarland 0.5, standard inoculum; and 2.5, large inoculum), and inhibition zones were read at 2-hour intervals from 10 hours to 24 hours [15].
†$P<0.05$, significant difference between the standard and large inoculums within a given category (i.e., readable N; % CA) and a given *Candida* species.
Abbreviations: BMD, broth microdilution; CA, categorical agreement; DDT, disk diffusion testing; ME, major error; Minor, minor error; VME, very major error.

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major error (ME) when an isolate classified as FS by the BMD method was classified as FR by DDT, or a minor error for all other discordances. The chi-square or Fisher's exact test was used to compare CA rates, and Student's *t*-test was used to compare the test time. Statistical analysis was performed using R version 4.0.3 (The R Project for Statistical Computing, Vienna, Austria), and significance was defined as $P<0.05$.

RESULTS

Comparison of standard DDT and BMD method results

Table 1 shows the CA rates between fluconazole susceptibility results obtained using the DDT and BMD methods for standard inoculum at 24 hours. The CA rates, determined using revised species-specific CBPs, between the two methods were 97.0%, 98.6%, 89.7%, and 95.8% for *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*, respectively. The overall CA rate for all 188 isolates was 96.3%, with a VME rate of 1.6% and an ME rate of 2.1%.

DDT results after short incubation

Table 3 shows the proportion (%) of readable isolates (forming uniformly circular inhibition zones with sufficient growth) of the four *Candida* species in DDT using the two inocula (McFarland 0.5 or 2.5) and their CA with the BMD method according to the incubation time (10–24 hours). Of the 188 isolates, the proportions of readable DDT results obtained using the standard inoculum were 35.1%, 63.8%, 92.0%, 94.7%, 96.3%, and 100% at 10, 12, 14, 16, 18, and 22 hours, respectively. The proportions of readable DDT results obtained using the large inoculum were 58.5%, 91.0%, 96.8%, and 100% at 10, 12, 14, and 16 hours, respectively. Overall, DDT using the standard inoculum produced readable results for >95% of *C. albicans*, *C. glabrata*, and *C. tropicalis* isolates at 14 hours, whereas results produced using the large inoculum were readable after 12 hours for *C. glabrata* and *C. tropicalis* and after 14 hours for *C. albicans* for >95% of the isolates. For *C. parapsilosis*, the proportion of readable DDT results using the standard inoculum was 65.5% at 14 hours, which was significantly lower than that observed for the large inoculum (89.7%, $P<0.05$). DDT results using the large inoculum were readable after 16 hours for >95% of *C. parapsilosis* isolates, whereas the results using the standard inoculum were readable after 20 hours for >95% of *C. parapsilosis* isolates.

Excellent CA (>90%) between DDT and the BMD method for assessing fluconazole susceptibility was observed for each incubation time tested for nearly all *C. albicans*, *C. glabrata*, and *C. tropicalis* isolates, irrespective of the inoculum size. For *C. parapsilosis*, the CA rates for DDT using the standard inoculum were 68.4%, 89.7%, and 89.7% at 14, 20, and 22 hours, whereas the CA rates for DDT using the large inoculum were >92% at all time points, with no VMEs; at 14 hours, the CA rate for DDT

Table 4. Fluconazole DDT using two inocula at the first readable time and their categorical agreement with the BMD reference method for 188 isolates of four common *Candida* species*

Species	N of isolates by fluconazole category by the BMD method				Inoculum used in DDT*	Mean (SD) of the first readable time (hr)	% CA	N (%) of isolates with discrepancies		
	FR	F-SDD	FS	Total				VME	ME	Minor
C. albicans	7	0	59	66	Standard	13.0 (2.2)	97.0	2 (3.0)	0 (0.0)	0 (0.0)
					Large	11.6 (1.6)†	100.0	0 (0.0)	0 (0.0)	0 (0.0)
C. glabrata	33	36	0	69	Standard	11.0 (1.6)	98.6	0 (0.0)	0 (0.0)	1 (1.4)
					Large	10.3 (0.8)†	98.6	0 (0.0)	0 (0.0)	1 (1.4)
C. parapsilosis	15	0	14	29	Standard	15.4 (2.3)	72.4	3 (10.3)	0 (0.0)	5 (17.2)
					Large	12.4 (1.5)†	96.6†	0 (0.0)	0 (0.0)	1 (3.4)
C. tropicalis	8	0	16	24	Standard	11.0 (1.4)	91.7	1 (4.2)	0 (0.0)	1 (4.2)
					Large	10.2 (0.6)†	95.8	1 (4.2)	0 (0.0)	0 (0.0)
Total	63	36	125	188	Standard	12.4 (2.5)	93.1	6 (3.2)	0 (0.0)	7 (3.7)
					Large	11.1 (1.5)†	98.4	1 (0.5)	0 (0.0)	2 (1.1)

*CLSI M27 BMD method was conducted strictly according to CLSI M27-ED4 document (24-hour incubation), but DDT was modified with the use of two cell suspensions (McFarland 0.5, standard inoculum; and 2.5, large inoculum), and inhibition zones were read at 2-hour intervals from 10 hours to 22 hours [15]. †$P<0.05$ between the standard inoculum and large inoculum within a given species.
Abbreviations: BMD, broth microdilution; CA, categorical agreement; DDT, disk diffusion testing; FR, fluconazole resistant; FS, fluconazole susceptible; F-SDD, fluconazole-susceptible dose-dependent; ME, major error; Minor, minor error; SD, standard deviation; VME, very major error.

Table 5. Distribution of inhibition zone diameters at the first readable time in fluconazole DDT using two inocula for 188 *Candida* isolates

Method/species	N of results at the following zone diameter (mm)																						Total N	Dis-tance*	Over-laps
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	≥27			
DDT using the standard (0.5 McFarland) inoculum																									
C. albicans (FS)														1		2	2	2	2	10	7	33	59	–8	27
C. albicans (FR)	2					1	1	1				1									1		7		
C. glabrata (F-SDD)						1					3	2	1	11	5	5	5	1		2			36	3	0
C. glabrata (FR)	32		1																				33		
C. parapsilosis (FS)															1			1	1	2	3	6	15	1	0
C. parapsilosis (FR)				1	1		4	1	3	2		1	1	1									15		
C. tropicalis (FS)													1			1	2	5	1		1	5	16	–1	2
C. tropicalis (FR)	3				1		1	1			1		1										8		
DDT using the large (2.5 McFarland) inoculum																									
C. albicans (FS)														3	2	3	3	6	5	6	4	27	59	13	0
C. albicans (FR)	7																						7		
C. glabrata (F-SDD)								1	1	2		1	4	6	5	6	2	3	2	2	1		36	5	0
C. glabrata (FR)	32		1																				33		
C. parapsilosis (FS)																1		2	2	6		3	14	5	0
C. parapsilosis (FR)	5					5		4			1												15		
C. tropicalis (FS)													1				2	2	4	2	1	4	16	1	0
C. tropicalis (FR)	2					3		2				1											8		

*Distance between the ranges for the wild-type and those for the FR variant populations for each species. A negative value indicates overlap between the two populations. The number of overlaps is calculated as the number of wild-type and FR variant isolates for which inhibition zone diameters were overlapping for each species.
Gray boxes indicate zone diameter breakpoints (mm) for FS, F-SDD, or FR in each species, according to the CLSI M60-ED2 document [16].
Abbreviations: DDT, disk diffusion testing; FR, fluconazole resistant; FS, fluconazole susceptible; F-SDD, fluconazole-susceptible dose-dependent.

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using the standard inoculum was significantly lower than that using the large inoculum (68.4% vs. 92.3%, $P<0.05$).

DDT results at first readable time

Table 4 shows the average first readable times for DDT for all 188 *Candida* isolates and the CA with the BMD method. The first readable times were significantly shorter for the large inoculum than for the standard inoculum for each of the four *Candida* species separately and all four species combined (all, $P<0.05$). The CA rates for DDT using the standard or large inoculum over the BMD method were similar for *C. albicans*, *C. glabrata*, and *C. tropicalis*. However, the CA rate for *C. parapsilosis* was lower for DDT using the standard inoculum than for DDT using the large inoculum ($P<0.05$). For the standard inoculum, three and five *C. parapsilosis* isolates with Y132F variants were misclassified as FS (VME rate, 10.3%) and F-SDD (minor error rate, 17.2%), respectively, at the first readable time.

Table 5 shows the distribution of DDT inhibition zone diameters obtained at the first readable time for the 188 *Candida* isolates using the standard or large inoculum. Wild-type and FR variant populations of *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* were separated by –8, 3, 2, and –1 mm, respectively, in tests using the standard inoculum, and by 13, 5, 5, and 1 mm, respectively, in tests using the large inoculum. Overall, the large inoculum resulted in better separation between FS and FR variant isolates than the standard inoculum, and an overlap was found for *C. albicans* and *C. tropicalis* isolates in tests using the standard inoculum.

DISCUSSION

With an increase in FR among *Candida* isolates, rapid fluconazole susceptibility testing has become more important, particularly in patients with invasive candidiasis. We showed that DDT using a large inoculum (McFarland 2.5) enabled faster assessment of FR in four common *Candida* species, with virtually no VME results on FR categorization, than DDT using a standard inoculum (McFarland 0.5).

The standardized DDT (CLSI M44-ED1 or ED2), which uses McFarland 0.5 inoculum and a 24-hour incubation period, has been extensively validated in comparison with the CLSI M27 BMD reference method for the assessment of fluconazole sensitivity in common *Candida* species [8, 13, 14]. However, none of the previous studies relying on the standardized DDT provided species-specific results, as they were performed before the development of revised species-specific CBPs for DDT and the CLSI M27 BMD method in 2010 [16].

In a large study of FR in 1,586 *Candida* isolates, the authors reported 93% CA, with VME and ME rates of only 0.1% and 0.3%, respectively [14]. The low VME rate (0.1%) in the previous study may be because FR isolates were rare in their collection (only 2.5% of isolates were FR) and partly because they used the previous CBP for the CLSI BMD method (MIC ≥64 μg/mL for all *Candida* species) [19]. In our study testing well-characterized isolates from a Korean collection, in which 33.5% of isolates were FR, the overall CA rate between the CLSI BMD method and DDT for all 188 isolates was 96.3%, with VME and ME rates of 1.6% and 2.1%, respectively. The present study revealed excellent CA between DDT (24-hour incubation) and the reference CLSI M27 BMD method using species-specific CBPs for fluconazole against *C. albicans* (97.0%), *C. glabrata* (98.6%), and *C. tropicalis* (95.8%); however, the CA for molecularly defined isolates of *C. parapsilosis* in the Korean collection was lower (89.7%).

To date, only three studies have directly evaluated DDT use for assessing fluconazole susceptibility in *Candida* species isolated from positive blood cultures [10, 11, 24]. These studies reported excellent CA between conventional (using colonies subcultured from positive blood cultures) and direct (using blood culture broth) DDT of fluconazole susceptibility [10, 11]. However, there were several potential shortcomings, including low numbers of antifungal-resistant isolates, the absence of earlier readings for DDT, and the lack of a direct comparison between DDT and the CLSI M27 BMD reference method. In the present study, *Candida* cell numbers in McFarland 0.5 and 2.5 inocula were $1.5–8.3\times10^6$ CFU/mL and $0.9–3.9\times10^7$ CFU/mL, respectively, with considerable variation among the four *Candida* species. Given that *Candida* cell numbers in positive blood cultures range from 10^5 to 10^8 CFU/mL, with most samples (87%) being in the range $10^6–10^7$ CFU/mL, we performed DDT using two cell suspensions, McFarland 0.5 (standard inoculum) and 2.5 (large inoculum) [11, 25].

DDT results were readable after 14 hours (*C. albicans*, *C. glabrata*, and *C. tropicalis*) and 20 hours (*C. parapsilosis*) for >95% of the isolates when the standard inoculum was used, compared with 12 hours (*C. glabrata* and *C. tropicalis*), 14 hours (*C. albicans*), and 16 hours (*C. parapsilosis*) when the large inoculum was used. Differences in the average first readable time between tests for DDT using standard and large inocula for *C. albicans* (13.0 hours vs. 11.6 hours), *C. glabrata* (11.0 hours vs. 10.3 hours), *C. parapsilosis* (15.4 hours vs. 12.4 hours), and *C. tropicalis* (11.0 hours vs. 10.2 hours) showed that DDT using large in-

oculum may save up to 1–3 hours compared with the traditional method. Thus, DDT using the large inoculum may enable rapid detection of FR isolates of all four common *Candida* species.

We found that DDT using both standard and large inocula produced readable results after 14 hours of incubation for >95% of *C. albicans*, *C. glabrata,* and *C. tropicalis* isolates. When the first readable DDT results were compared with those produced using the CLSI M27 BMD method, the CA rates was 97.0%, 98.6%, and 91.7% for *C. albicans*, *C. glabrata*, and *C. tropicalis*, respectively, when the standard inoculum was used and 100%, 98.6%, and 95.8%, respectively, when the large inoculum was used. Overall, the CA between the methods was excellent (>90%) for all three species across all time points (14–22 hours). However, VMEs were found for two (3%) *C. albicans* isolates in DDT using the standard inoculum at the first readable time, while the U.S. Food and Drug Administration requires <1.5% of resistant isolates evaluated to yield VMEs [26]. One FR isolate of *C. albicans* with a G464S variant in Erg11p with a fluconazole MIC of 8 g/mL was consistently associated with VME (inhibition zone diameter, 17.25–18.27 mm, reading time, 14–24 hours) in DDT using the standard inoculum, but showed a decrease in inhibition zone diameter (6.0–17.3 mm) in DDT using the large inoculum, in which most VMEs changed into minor errors. The other FR isolate of *C. albicans* with a K143R variant in Erg11p and a fluconazole MIC of 8 g/mL yielded a VME in DDT using both standard and large inocula, which may be attributed to the poor growth of the isolate on Mueller-Hinton agar. In addition, of the 15 FR isolates of *C. parapsilosis* harboring the Y132F variant in Erg11p, three isolates with a fluconazole MIC of 8 g/mL yielded a VME in DDT using the standard inoculum at an earlier reading time, although all three isolates exhibited a decrease in inhibition zone diameter after longer incubation. This is likely due to the relatively slow growth of *C. parapsilosis* on RPMI-glucose medium, suggesting that earlier reading of DDT results when the standard inoculum is used is unlikely during direct antifungal susceptibility testing of *C. parapsilosis* for fluconazole using DDT. Taken together, these results suggest that DDT using the large inoculum can be successfully used to test fluconazole susceptibility rapidly, which may lead to more appropriately targeted antifungal drug therapy and better outcomes in patients with candidemia due to common *Candida* species, especially *C. parapsilosis.*

When inhibition zone diameters at the first reading for each isolate were recorded, DDT using the standard inoculum showed overlap between wild-type isolates and FR variant populations for both *C. albicans* and *C. tropicalis*. However, a clear separation between wild-type and FR variant isolates was observed for all isolates of *C. glabrata* (standard inoculum, 3 mm; large inoculum, 5 mm), and *C. parapsilosis* (standard inoculum, 1 mm; large inoculum, 5 mm) in DDT using both standard and large inocula, suggesting that DDT may be a promising tool for the rapid differentiation of FR variants from wild-type isolates of *C. glabrata and C. parapsilosis*, irrespective of the inoculum size. However, further studies using more isolates are needed to establish new breakpoints for rapid DDT for each *Candida* species by considering equivalent MIC values, pharmacokinetic and pharmacodynamic parameters, resistance mechanisms, and clinical outcomes, as these parameters relate to the DDT breakpoint values.

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AUTHOR CONTRIBUTIONS

Shin JH designed the study; Jeon S performed laboratory measurements; Lim HJ, Choi MJ, Byun SA, and Lee D collected and identified clinical isolates, and performed molecular studies; Shin JH, Won EJ; and Jeon S wrote the preliminary manuscript; Shin JH, Jeon S, and Lim HJ analyzed the data; Shin JH revised the manuscript; Lee SY, Won EJ, Kim SH, and Shin MG provided valuable comments and recommendations. All authors revised and accepted the final version of the manuscript.

CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article were reported.

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ORCID

Suhak Jeon https://orcid.org/0000-0002-9129-132X
Jong Hee Shin https://orcid.org/0000-0001-9593-476X
Ha Jin Lim https://orcid.org/0000-0003-2436-5007

Min Ji Choi https://orcid.org/0000-0001-8576-4015
Seung A Byun https://orcid.org/0000-0003-4013-7274
Dain Lee https://orcid.org/0000-0003-0236-9808
Seung Yeob Lee https://orcid.org/0000-0003-2851-1112
Eun Jeong Won https://orcid.org/0000-0002-8750-4257
Soo Hyun Kim https://orcid.org/0000-0001-9739-711X
Myung Geun Shin https://orcid.org/0000-0002-0372-9185

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