Received: 11 May 2017 | Accepted: 13 June 2017

DOI: 10.1002/jcla.22297

RESEARCH ARTICLE

WILEY

Evaluation of direct antifungal susceptibility testing methods of *Candida* spp. from positive blood culture bottles

Yasemin Oz | **Egemen Gokbolat**

Faculty of Medicine, Department of Microbiology, Eskisehir Osmangazi University, Eskisehir, Turkey

Correspondence
Yasemin Oz, Faculty of Medicine, Department of Microbiology, Division of Mycology, University of Eskisehir Osmangazi, Eskisehir, Turkey.
Email: dryaseminoz@gmail.com

Present Address:
Egemen Gokbolat, Department of Microbiology, Burdur State Hospital, Burdur, Turkey

Funding information
Eskisehir Osmangazi University Commission Research Projects, Grant/Award Number: 2014-4238

Background: Blood culture is the gold standard diagnostic method in candidemia in spite of long result time and low sensitivity rate. Early diagnosis is crucial for management of candidemia because a delay in treatment is related with increased mortality. We aimed to evaluate the direct applicability of antifungal susceptibility testing methods from positive blood culture bottles to save at least 24 hours.

Methods: Blood culture bottles were inoculated with 62 *Candida* isolates. Etest and broth microdilution (BMD) methods for six antifungals, disk diffusion (DD) method for two antifungals were performed, both directly from bottles and standardly.

Results: Essential agreements between direct and standard Etest methods were 87.1% for caspofungin and >90% for other antifungals, but the agreements of them with reference BMD were relatively low. Essential agreement between direct and standard BMD was >93%. Correlation between direct and standard DD methods was very high, negative correlations were observed between reference BMD and DD methods.

Conclusion: BMD is a reference method to evaluate the antifungal susceptibility, direct application of BMD might provide reliable results at least 24 hours earlier. Direct DD method may be a qualitative alternative. Direct susceptibility testing methods may be very useful to initiating the appropriate treatment on time.

KEYWORDS

blood culture, broth microdilution, *Candida*, direct antifungal susceptibility, disk diffusion, Etest

1 | INTRODUCTION

Bloodstream infections (BSI) caused by *Candida* species are significant causes of morbidity and mortality, especially in intensive care unit patients and patients with hematological malignancy. Although *Candida* spp. were the fourth most common pathogens isolated from blood cultures, the crude mortality rate of *Candida* infections was the highest among all nosocomial BSI.[1] Therefore, rapid and accurate diagnostic methods are essential for appropriate management of *Candida* BSIs. However, blood culture is still the gold standard diagnostic method in spite of long resulting time and low sensitivity rate in candidemia. The standard method of blood culture involves at least overnight agar medium subculture and then identification and antifungal susceptibility testing after a bottle is signaled as positive for *Candida*; it is time consuming and can delay the appropriate therapy. Several standard methods were suggested for antifungal susceptibility testing of yeast by both Clinical and Laboratory Standards Institute—CLSI (M27-A3 and M44-A2) and European Committee on Antimicrobial Susceptibility Testing—EUCAST (E.DEF 7.2). Besides, there are many commercial manual or automated systems such as Etest, Sensititre YeastOne, and Vitek which are used commonly in many clinical laboratories.

Several studies including direct inoculation from positive blood culture bottles have been performed in order to decrease the time required for identification and/or antifungal susceptibility testing methods.[2–7] In these studies, researchers reported promising results by Etest, disk diffusion, flow cytometry, and some commercial systems (Sensititre YeastOne and Vitek 2). Thus, antifungal susceptibility results could be obtained about 24 hours earlier than those obtained with the standard procedure. Early start of proper antifungal treatment can significantly improve the outcome of patients with candidemia because the mortality rate increases almost 1.5% for each hour in delay of antifungal treatment.[8]

Standard yeast stock suspension using in all antifungal susceptibility methods needs 0.5 McFarland turbidity including 1-5×10^6 cells/mL.[9,10] Although the initial fungal burden was very low in over half of all *Candida* blood cultures (>50% of cultures had ≤1 cell/mL),[11] Chang et al.[4] showed that *Candida* cell counts in positive blood culture bottles were in the range of 10^5 to 10^8 cells/mL, with 87% of bottles including 10^6-10^7 cells/mL (almost equivalent to 0.5 McFarland standard). These researchers showed that variations in the yeast cell number in the blood culture bottle had no impact on the minimal inhibitory concentration (MIC).[4] Eventually, directly blood-yeast-broth mixtures in positive blood culture bottles were actively used as a fungal stock suspension for direct Etest and disk diffusion susceptibility methods in previous studies.[4,5,12]

In this study, we aimed to evaluate the direct applicability of standard disk diffusion test, a commercial Etest and broth microdilution method from positive blood culture bottles for six antifungals. Differently from most previous reports,[3–6] we used the blood culture bottles inoculated with a standardized inoculum size of the most common five species of candidemia. Furthermore, the direct applicability of reference broth microdilution test from positive blood culture bottle has been evaluated for the first time in this study. We also used the fungal suspensions in positive blood culture bottles as stock suspension for all direct methods in our study.

2 | MATERIALS AND METHODS

2.1 | Isolates

A total of 60 clinical bloodstream isolates of *Candida* (*C. albicans* [n=20], *C. parapsilosis* [n=10], *C. tropicalis* [n=10], *C. glabrata* [n=10], and *C. krusei* [n=10]) and two quality control isolates (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019) were included in this study. The identification of all clinical isolates had been made previously by using a commercial assimilation test (API 20C AUX, bioMérieux, Marcy l'Etoile, France).

This study was approved by the Clinical Research Ethics Committee of Eskisehir Osmangazi University with the number of 19 at December 06, 2013.

2.2 | Preparation of blood culture bottles

BACTEC Plus Aerobic/F culture vials (BD Diagnostics, Sparks, MD) were inoculated with 8 mL fresh whole blood samples of healthy human volunteers according to the manufacturer's recommendations. Simultaneously, standard 0.5 McFarland yeast suspension was prepared and diluted with sterile saline for each isolate. Final inoculums were adjusted to be 1000-5000 yeast cells in each bottle. All bottles were incubated at 35°C with continuous agitation in the BACTEC 9240 (BD Diagnostics, Sparks, MD) blood culture instrument. After the bottles signaled as positive, direct susceptibility testing methods were performed at once. In addition, all positive bottles were also subcultured onto Sabouraud dextrose agar (SDA) plates and incubated for 18-24 hours at 37°C for standard susceptibility testing methods.

2.3 | Colony counting

For predictability of the cell numbers in the bottles, six positive blood culture bottles were randomly selected, agitated. Then 1 mL sample was aspirated from each of these bottles, serially 10-fold diluted with sterile saline and 50 μL of suspensions was plotted on potato dextrose agar plate. After 48 hours incubation, colony counting was made.

2.4 | Antifungal susceptibility testing methods

2.4.1 | Standard and direct disk diffusion methods

Disk diffusion (DD) testing was performed using fluconazole (FLU, 25 μg) and voriconazole (VOR, 1 μg) disks (Becton Dickinson, Sparks, MD) as described in CLSI M44-A2.[9] For standard disk diffusion testing, inoculums were prepared by picking 4-5 colonies from 24-hour cultures on SDA. Colonies were suspended in 5 mL of sterile saline and turbidity was adjusted to 0.5 McFarland. Final concentrations of the inoculums were 1-5×10^6 CFU/mL. A sterile cotton swab was dipped into the suspension and excess fluid was removed. The inoculum was spread by evenly streaking the swab onto surface of Mueller-Hinton agar (Difco Laboratories) supplemented with 2% glucose and 0.5 μg/mL methylene blue and the plates were dried at 35°C for 15 minutes prior to placing the antifungal disks. After incubation at 35°C for 48 hours, the inhibition diameters around the disks were measured. For direct disk diffusion testing, the sample was directly aspirated into a sterile tube after shaking the positive blood culture bottle. A sterile cotton swab was dipped into this suspension and subsequent steps were performed as standard disk diffusion testing described above.

The inhibition zones of DD testing were measured in the area that showed a sharp decrease in growth density. The interpretive criteria for DD testing were published by the CLSI: for all species against VOR, the susceptible (S) inhibition zone diameter was ≥17 mm, the susceptible dose-dependent (SDD) inhibition zone diameter was 14-16 mm, and the resistant (R) inhibition zone diameter was ≤13 mm. The zone diameters of FLU ≥19 mm were interpreted as S, 15-18 mm as SDD, and ≤14 mm as R for *C. albicans*, *C. parapsilosis*, and *C. tropicalis*, and ≥15 mm was interpreted as S-DD and ≤14 mm as R for *C. glabrata*.[5,9]

2.4.2 | Standard and direct Etest methods

FLU, VOR, posaconazole (POS), caspofungin (CAS), anidulafungin (AND), and amphotericin B (AMB) Etest strips were purchased from manufacturer (AB Biodisk, Solna, Sweden). Standard Etest method was performed according to the manufacturer's instructions. A sterile cotton swab was used to inoculate the 0.5 McFarland turbidity yeast suspension onto a 150-mm agar plate containing RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 2% glucose and buffered with morpholinepropanesulfonic acid (MOPS, Sigma) to pH 7.0. After the excess moisture was fully absorbed into the agar, Etest strips were placed onto inoculated agar. Plates were incubated at 35°C for 48 hours. MICs were read as the lowest concentration at which the growth inhibition zone intersected the Etest strip, ie,

complete inhibition for AMB and 80% inhibition for others after 24 and 48 hours of incubation.[13] Distinctively for direct Etest method, inoculation of agar plates was made by directly aspirated samples from agitated positive bottles. Other steps were performed as standard Etest method described above.

2.4.3 | Standard and direct broth microdilution methods

Standard broth microdilution (BMD) method was performed by strictly following the reference microdilution M27-A3 procedure of the CLSI[10] and standard BMD was the reference antifungal susceptibility method in this study. Antifungal stock solutions were prepared in distilled water for FLU and CAS, in dimethyl sulfoxide for VOR, POS, AMB, and AND. Serial twofold final dilutions of all drugs were prepared in RPMI 1640 medium buffered with MOPS. The final concentrations in 96-well microtiter plates were 0.06-64 mg/L for FLU, 0.03-16 mg/L for VOR, POS, and AMB, and 0.015-8 mg/L for CAS and AND. All isolates were subcultured onto SDA. After 24- to 48-hour incubation, standard 0.5 McFarland fungal suspensions were prepared with sterile 0.85% saline. They were diluted with RPMI 1640 medium to obtain a starting inoculum which results in $1\text{-}5\times10^3$ cells/mL. Microtiter plates were inoculated and incubated at 35°C. The MICs were read at 24 and 48 hours. Distinctively for the direct BMD methods, directly aspirated samples from positive bottles were used. They were diluted 1:50, and further diluted 1:20 with RPMI 1640 medium to obtain the starting inoculum. All of other steps were performed as standard method described above. Endpoints for azoles and echinocandins were defined as the lowest concentration of drug that resulted in a prominent reduction (approximately 50% inhibition) of visual growth compared with the growth control wells. Endpoint of AMB was defined as the lowest concentration of drug which resulted in total inhibition of visual growth.

2.5 | Analysis of results

The results obtained from each direct method were compared to results of their own standard methods in terms of both categorical and essential agreement. In addition, the results of standard disk diffusion and Etest methods were compared to reference broth microdilution results.

Species-specific clinical breakpoints were described in CLSI M27-S4 guide for five common *Candida* spp. and FLU, VOR, and echinocandins.[14] However, no approved interpretive breakpoints are already available for POS and AMB. Categorical agreements and discrepancies between the standard and direct methods were evaluated depending on the existence of interpretative breakpoints. Categorical agreement was defined for the isolates classified in the same category by both methods, and discrepancies between the standard and direct methods were defined as very major, major, and minor errors. Very major error (VME) was considered for a susceptible isolate by direct method but resistant by standard method and major error (ME) was reported for a resistant isolate by direct method but susceptible by standard method. Minor error (MiE) was considered for a susceptible or resistant isolate by direct method and intermediate by standard method or vice versa.[3,15]

In the situations that lack breakpoints, discrepancies were classified as nonsubstantial and substantial differences as described by Cuenca-Estrella et al.[15]; nonsubstantial differences (NSD) were defined as discrepancies in MIC results of three or four twofold dilutions, and substantial differences (SD) were defined as discrepancies of more than four twofold dilutions. Essential agreement was evaluated for the methods determined MIC values; discrepancies of no more than ±2 dilutions were used to calculate the percent agreement.[15] Etest MICs were rounded up to the nearest even log2 concentration to facilitate the comparison with broth microdilution results.

Acceptable percent of essential agreement for MICs was decided as ≥90% for each antifungal agent against all organisms and an acceptable overall categorical agreement was set at ≤1.5% VME and ≤3% ME.[13]

2.6 | Statistical analysis

Descriptive statistics were calculated using Microsoft Excel. The data were evaluated by using the Statistical Package for Social Sciences (SPSS) version 20.0 statistical package program (IBM Corp, Armonk, NY, USA). Paired-sample *t* test was used to evaluate the correlations and differences between MICs obtained by the direct and standard test methods. A correlation coefficient of ≥0.8 was considered very strong correlation, 0.6-0.79 strong, 0.4-0.59 moderate, 0.2-0.39 weak, and ≤0.19 as very weak.[5] A P of ≤.05 was considered statistically significant for all tests.

3 | RESULTS

The colony counts of yeasts in six randomly selected positive blood bottles ranged from $8\times10^5\text{-}3\times10^7$ CFU/mL. The results of direct and standard antifungal susceptibility testing methods were summarized in Table 1. When the methods were evaluated within themselves, the results obtained from direct and standard tests for each method were found similar in general; MIC50 and MIC90 values were within ±2 dilutions ranges for each isolate. The most prominent difference was observed for FLU and VOR disk diffusion tests against *C. glabrata*; zone diameters were larger in standard method than direct method.

When the Etest method was performed directly from positive blood culture bottles, essential agreements with standard Etest method were 87.1% for CAS and >90% for other antifungals (Table 2). Five VMEs (8%) in CAS, one SD (1.6%) in POS, and one ME (1.6%) in VOR were detected. The MIC results of all antifungals for more than half of all isolates were in ±1 dilution ranges by standard Etest method. Direct Etest results were highly compatible with standard Etest method (P<.001) and any significant difference between mean MIC values of direct and standard Etest methods was not found for all drugs (P>.05). When the direct and standard Etest methods were compared to reference BMD method, essential agreements were lower (67%-79% for

TABLE 1 The results of direct and standardized antifungal susceptibility testing methods

Isolates and antifungals	Etest (μg/mL)						Broth microdilution test (μg/mL)						Disk diffusion test (mm)			
	Direct			Standardized			Direct			Standardized			Direct		Standardized	
	MIC range	MIC50	MIC90	MIC range	MIC50	MIC90	MIC range	MIC50	MIC90	MIC range	MIC50	MIC90	Zone range	Mean	Zone range	Mean
C. albicans (n=20)																
Fluconazole	0.032-2	0.25	0.75	0.064-0.5	0.19	0.5	0.06-0.25	0.125	0.125	0.06-0.25	0.125	0.25	30-50	36.15	30-45	37.05
Voriconazole	0.012-0.125	0.032	0.064	0.012-0.064	0.032	0.047	0.03-0.06	0.03	0.03	0.03	0.03	0.03	32-60	39.7	30-50	38.95
Posaconazole	0.002-0.125	0.023	0.125	0.006-0.125	0.023	0.094	0.03-0.06	0.03	0.03	0.03	0.03	0.03				
Caspofungin	0.002-0.5	0.125	0.25	0.002-1	0.25	0.38	0.015	0.015	0.015	0.015-0.06	0.03	0.06				
Anidulafungin	0.002-0.064	0.023	0.064	0.002-0.064	0.023	0.032	0.015	0.015	0.015	0.015	0.015	0.015				
Amphotericin B	0.047-3	0.38	1	0.047-3	0.38	0.75	0.25-1	0.25	0.5	0.125-1	0.25	0.5				
C. glabrata (n=10)																
Fluconazole	2-256	24	64	2-256	32	256	2-64	8	16	4-64	8	32	6-24	14.4	6-28	18.6
Voriconazole	1-32	1.5	3	1-32	2	3	0.125-16	0.5	2	0.25-4	0.5	1	6-26	16.5	6-28	20.5
Posaconazole	0.064-32	32	32	0.5-32	32	32	0.125-16	1	16	0.25-16	0.5	16				
Caspofungin	0.047-0.25	0.125	0.25	0.002-0.75	0.25	0.5	0.015-0.06	0.03	0.03	0.06-0.25	0.06	0.06				
Anidulafungin	0.008-0.19	0.125	0.125	0.008-0.19	0.094	0.19	0.015	0.015	0.015	0.015-0.03	0.015	0.03				
Amphotericin B	0.125-8	0.5	1.5	0.064-8	0.5	6	0.25-1	0.5	1	0.25-1	0.5	1				
C. parapsilosis (n=11)																
Fluconazole	0.125-2	0.75	1.5	0.064-4	0.5	3	0.125-2	0.5	1	0.06-2	0.5	1	15-44	27.09	15-40	27.18
Voriconazole	0.023-0.38	0.047	0.38	0.023-0.75	0.047	0.25	0.03-006	0.03	0.06	0.03-006	0.03	0.06	22-46	30.45	22-40	31.72
Posaconazole	0.016-0.38	0.125	0.19	0.008-0.25	0.032	0.094	0.03	0.03	0.03	0.03	0.03	0.03				
Caspofungin	0.25-4	0.38	2	0.125-3	0.5	2	0.015-0.125	0.06	0.125	0.125-0.5	0.25	0.5				
Anidulafungin	0.25-8	0.75	4	0.25-6	0.5	3	0.06-1	0.25	1	0.125-1	0.5	1				
Amphotericin B	0.094-32	0.5	0.75	0.032-32	0.38	1	0.25-1	0.5	1	0.06-0.5	0.5	0.5				
C. tropicalis (n=10)																
Fluconazole	0.25-4	1	4	0.38-4	1	3	0.06-2	0.5	0.5	0.125-0.5	0.25	0.5	6-30	20.6	10-34	23.5
Voriconazole	0.125-1	0.38	0.5	0.023-0.75	0.125	0.25	0.03-0.5	0.06	0.125	0.03-0.15	0.03	0.06	6-32	18.8	15-30	22.5
Posaconazole	0.094-0.38	0.19	0.25	0.047-0.5	0.125	0.38	0.03-0.06	0.03	0.03	0.03	0.03	0.03				
Caspofungin	0.094-0.38	0.19	0.38	0.002-0.38	0.38	0.38	0.015	0.015	0.015	0.015-0.06	0.06	0.06				
Anidulafungin	0.006-0.094	0.064	0.094	0.008-0.064	0.032	0.047	0.015	0.015	0.015	0.015	0.015	0.015				
Amphotericin B	0.25-12	1.5	3	0.5-16	1.5	3	0.25-1	0.5	1	0.25-1	0.5	1				
C. krusei (n=11)																
Fluconazole	3-128	24	96	3-128	32	128	8-64	32	32	16-32	32	32	6-12	7.27	6-14	8.36
Voriconazole	0.25-1	0.5	1	0.25-2	0.75	2	0.25-2	0.5	1	0.06-1	0.5	1	15-26	19.63	16-25	20.36
Posaconazole	0.38-3	0.5	0.5	0.19-0.5	0.38	0.5	0.03-0.25	0.125	0.125	0.03-1	0.25	0.25				
Caspofungin	0.094-0.5	0.19	0.38	0.012-0.5	0.094	0.38	0.06-0.25	0.06	0.25	0.06-0.5	0.25	0.25				
Anidulafungin	0.008-0.125	0.016	0.094	0.008-0.094	0.023	0.064	0.015-0.06	0.015	0.06	0.015-0.06	0.015	0.03				
Amphotericin B	1-32	12	32	0.5-32	8	32	0.5-1	1	1	0.5-1	1	1				

direct, 50%-85% for standard Etest methods) (Table 3). VME was not observed for both Etest methods, but ME and SD rates were higher in standard Etest than direct Etest in comparison to reference BMD method. Although the correlation coefficients for both Etest methods with reference BMD method were lower, correlations were significant ($P<.05$, Table 3). In addition, differences between mean MIC values of reference BMD and both Etest methods were found significant for CAS, POS, and AMB ($P\leq.05$).

When the BMD method was performed from positive blood culture bottles directly, essential agreement with standard BMD method was very high (93.5% for CAS, >98% for other antifungals), VME and SD were not observed, only one ME (1.6%) was detected (Table 2). The MIC results of 56 isolates for FLU, 52 isolates for VOR, 60 isolates for POS and AND, 29 isolates for CAS, and 57 isolates for AMB were found in ±1 dilution ranges by standard BMD method. Direct BMD test results were highly compatible with standard BMD ($P<.001$), significant differences were only found among mean MIC values for CAS and AND ($P<.001$ and $P=.032$).

Direct and standard disk diffusion methods were performed only for FLU and VOR in this study. When this method was performed directly, ME was observed in VOR for two isolates (3.2%) and VME did not occur. Correlation between direct and standard disk diffusion methods was very high ($P<.001$, Table 2), although the differences between mean zone diameters of direct and standard methods were significant ($P=.005$ for FLU, $P=.43$ for VOR). When the results of direct and standard disk diffusion methods were evaluated according to reference BMD, negative correlations were observed for both antifungals ($P<.001$, Table 3). VME was not detected; a few MEs were observed for FLU in both direct and standard disk diffusion and for VOR in only direct method.

4 | DISCUSSION

The variability in agent spectrum and susceptibility characteristics of *Candida* spp. increases in parallel with increasing fungal infection rate. Early diagnosis is crucial for management in time of candidemia; it has been reported that each 1 hour delay in starting antifungal treatment results in increasing mortality almost 1.5%.[8] Therefore, earlier and appropriate treatment is very important to prevent increased mortality. In this study, we evaluated the direct applicability of common antifungal susceptibility methods to save at least 24 hours.

Etest method is an extremely easy, agar-based concentration gradient procedure for determining the MIC values and it has a good correlation (>90%) with reference methods.[13,15] But it is an expensive commercial test at least for our country. The agreements between direct and standard Etest methods have been found 100% for FLU, VOR, AMB and 86.21% for CAS in a recent study.[5] In an earlier study, when direct and standard Etest methods were compared to reference BMD method, relatively lower agreement rates have been observed; 81.8 and 89.9% for AMB, 89.4 and 78.3% for FLU, respectively.[4] Guinea et al.[12] evaluated 328 yeast isolates including *Candida* and non-*Candida* species against six antifungal drugs, they reported

TABLE 2 Agreement between direct and standardized susceptibility testing methods

	Etest							Broth microdilution							Disk diffusion					
	Categorical agreement (n)					Essential agreement		Categorical agreement (n)					Essential agreement		Categorical agreement (n)					
Antifungals	VME	ME	MiE	SD	NSD	(%)	CC	*P*	VME	ME	MiE	SD	NSD	(%)	CC	*P*	VME	ME	MiE	CC	*P*
Fluconazole	0	0	5			95.2	0.786	<.001	0	0	0			100	0.913	<.001	0	0	5	0.931	<.001
Voriconazole	0	1	3			95.2	0.996	<.001	0	1	5			100	0.897	<.001	0	2	6	0.889	<.001
Posaconazole				1	5	90.3	0.910	<.001				0	1	98.4	0.998	<.001					
Caspofungin	5	0	13			87.1	0.776	<.001	0	0	2			93.5	0.589	<.001					
Anidulafungin	0	0	5			96.8	0.591	<.001	0	0	0			100	0.936	<.001					
Amphotericin B				0	0	100	0.992	<.001				0	1	98.4	0.578	<.001					

VME, very major errors; ME, major errors; MiE, minor errors; SD, substantial differences; NSD, nonsubstantial differences; CC, paired samples correlation coefficients between direct and standardized methods; *P* values of ≤.05 indicate significant agreement.

TABLE 3 Agreement of direct and standardized susceptibility testing methods with reference broth microdilution method

Antifungals	Etest (direct and standardized)															Disk diffusion (direct and standardized)											
	Categorical agreement (n)									Essential agreement (%)		CC		P		Categorical agreement (n)								CC		P	
	VME		ME		MiE		SD		NSD							VME		ME		MiE							
	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST	DT	ST
Fluconazole	0	0	0	0	4	7					79.0	77.4	0.765	0.510	<.001	<.001	0	0	1	1	6	2	−0.695	−0.734	<.001	<.001
Voriconazole	0		1	2	15	10					77.4	85.5	0.896	0.898	<.001	<.001	0	0	2	0	4	4	−0.458	−0.492	<.001	<.001
Posaconazole							5	7	15	7	67.7	77.4	0.614	0.613	<.001	<.001										
Caspofungin	0	0	0	0	9	17					74.2	50.0	0.489	0.361	<.001	.004										
Anidulafungin	0	0	1	0	2	5					67.7	83.9	0.505	0.709	<.001	<.001										
Amphotericin B							7	7	9	11	74.2	71.0	0.432	0.423	<.001	.001										

VME, very major errors; ME, major errors; MiE, minor errors; SD, substantial differences; NSD, nonsubstantial differences; CC paired samples correlation coefficients between direct/standardized Etest/disk diffusion and reference methods; *P* values of ≤.05 indicate significant agreement.

that while the total essential agreements between direct and standard Etest methods were the lowest (69.5%) for AMB and the highest (94.2%) for CAS, between direct Etest and BMD methods were the lowest (55.2%) for POS and the highest (89.3%) for CAS, and between standard Etest and BMD methods were the lowest (63%) for FLU and the highest (91.5%) for CAS. As seen that, although it can differ according to species and drugs, the agreement rates of both direct and standard Etest with reference BMD were relatively low. Similarly to these reports, although we observed high correlation between direct and standard Etest methods except for CAS, the agreements of both direct and standard Etest methods with reference BMD method were <90% (67%-79% and 50%-86%, respectively) in this study (Table 3). In this case, it is difficult to reach a definitive conclusion; if the acceptable percent of essential agreement is considered as ≥90% for each antifungal,[13] Etest method (both direct and standard) will be below acceptable percent; however, if the Etest is approved as a potential antifungal susceptibility testing method, its direct application may be a convenient procedure because of high correlation between the results of direct and standard Etest methods.

Relatively low correlation between direct and standard Etest methods for CAS was remarkable in our study. A supportive result has been reported Jabeen et al.,[5] the lowest categorical agreement rate was detected between direct and standard Etest methods of CAS. This may be explained by the significant interlaboratory variation in caspofungin; Espinel-Ingroff et al.[16] compared both CLSI CAS MICs for 145-11 550 *Candida* isolates from 17 laboratories and EUCAST CAS MICs for 403-2556 isolates from seven laboratories, they observed an exceptional amount of MIC variability among participant laboratories.

Broth microdilution method has been standardized by CLSI and widely used as both reference and routine antifungal susceptibility testing method, although it is labor intensive and time consuming. We performed the BMD method by using positive blood culture broth directly as a fungal stock suspension without further pretreatment as reported previously.[4,5,12] The agreement between direct and standard BMD methods was in acceptable percent (essential agreement >93%, ME<3%, and no VME). In addition, the correlation of direct and standard methods was also excellent statistically, the MIC values of more than 83% of isolates were within ±1 dilution range except for CAS; CAS MICs of almost half of all isolates were within ±1 dilution range. These results support the study of Avolio et al.;[3] they reported 98% agreement between direct and standard procedures from positive blood culture bottles by using a commercial BMD test. However, we represented an easier and cheaper method in our study because we did not make any pretreatment for the preparation of stock fungal suspensions and we used in-house prepared microplates. To our knowledge, this study is important because of the first study evaluating the direct applicability of reference BMD method.

The DD method is another agar-based procedure as easy as Etest to perform in the clinical laboratories and it has been standardized by CLSI for FLU and VOR against common *Candida* species.[9] DD method has been evaluated directly from positive blood culture bottles by Jabeen et al.[5] and the agreements between direct and standard

methods have been reported as 97%-100% for FLU, VOR, and AMB. In another study, FLU DD test with direct subcultured chromogenic agar from positive blood culture bottle yielded 90%-100% agreement compared to BMD method at 24 hours; but 52% agreement with 10 MiE and one VME for only *C. glabrata* isolates.[2] This is an interesting approach because chromogenic medium is not standardized and not included in any test guide. We observed high correlation between direct and standard DD methods even though the differences between mean zone diameters of direct and standard methods were significant. In addition, the correlations between both DD methods and reference BMD method were good, no VME and very few MEs were detected. However, DD is a qualitative method; it cannot give any data about MIC values of drugs.

In conclusion, although the agreement between direct and standard Etest methods was excellent, the agreement of them with reference BMD was relatively low. Therefore, the use of both direct and standard Etest method can be problematic. BMD is a reference antifungal susceptibility method and its direct application gave excellent agreement rates. Thus, direct BMD method might provide reliable results at least 24 hours earlier. Direct DD method may be a qualitative alternative to BMD because the correlations between DD methods and reference BMD method were good. Direct antifungal susceptibility testing methods may be very useful to initiation of the appropriate treatment on time. However, these tests provide the preliminary susceptibility results, which should be confirmed by standard methods of tests.

ACKNOWLEDGMENTS

This work was supported by Eskisehir Osmangazi University Commission Research Projects (BAP, No. 2014-4238).

REFERENCES

1. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis*. 2004;39:309-317.
2. Tan GL, Peterson EM. CHROMagar *Candida* medium for direct susceptibility testing of yeast from blood cultures. *J Clin Microbiol*. 2005;43:1727-1731.
3. Avolio M, Grosso S, Bruschetta G, De Rosa R, Camporese A. Direct antifungal susceptibility testing of positive *Candida* blood cultures by sensititre YeastOne. *New Microbiol*. 2009;32:179-184.
4. Chang HC, Chang JJ, Chan SH, et al. Evaluation of Etest for direct antifungal susceptibility testing of yeasts in positive blood cultures. *J Clin Microbiol*. 2001;39:1328-1333.
5. Jabeen K, Kumar H, Farooqi J, Mehboob R, Brandt ME, Zafar A. Agreement of Direct Antifungal Susceptibility Testing from Positive Blood Culture Bottles with the Conventional Method for *Candida* Species. *J Clin Microbiol*. 2016;54:343-348.
6. Idelevich EA, Grunewald CM, Wüllenweber J, Becker K. Rapid identification and susceptibility testing of *Candida* spp. from positive blood cultures by combination of direct MALDI-TOF mass spectrometry and direct inoculation of Vitek 2. *PLoS ONE*. 2014;9:e114834.
7. Rudensky B, Broide E, Berko N, Wiener-Well Y, Yinnon AM, Raveh D. Direct fluconazole susceptibility testing of positive *Candida* blood cultures by flow cytometry. *Mycoses*. 2008;51:200-204.
8. Morrell M, Fraser VJ, Kollef MH. Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. *Antimicrob Agents Chemother*. 2005;49:3640-3645.
9. Clinical and Laboratory Standards Institute. *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline*. 2nd edn, M44-A2 Wayne, PA: Clinical and Laboratory Standards Institute; 2009.
10. Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard*. Third Edition. CLSI document M27-A3. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
11. Pfeiffer CD, Samsa GP, Schell WA, Reller LB, Perfect JR, Alexander BD. Quantitation of *Candida* CFU in initial positive blood cultures. *J Clin Microbiol*. 2011;49:2879-2883.
12. Guinea J, Recio S, Escribano P, et al. Rapid antifungal susceptibility determination for yeast isolates by use of Etest performed directly on blood samples from patients with fungemia. *J Clin Microbiol*. 2010;48:2205-2212.
13. Alexander BD, Byrne TC, Smith KL, et al. Comparative evaluation of Etest and sensititre yeastone panels against the Clinical and Laboratory Standards Institute M27-A2 reference broth microdilution method for testing *Candida* susceptibility to seven antifungal agents. *J Clin Microbiol*. 2007;45:698-706.
14. Clinical and Laboratory Standards Institute. *Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Fourth Informational Supplement M27-S4*. CLSI, Wayne, PA: Clinical and Laboratory Standards Institute; 2012.
15. Cuenca-Estrella M, Gomez-Lopez A, Alastruey-Izquierdo A, et al. Comparison of the Vitek 2 antifungal susceptibility system with the clinical and laboratory standards institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Broth Microdilution Reference Methods and with the Sensititre YeastOne and Etest techniques for in vitro detection of antifungal resistance in yeast isolates. *J Clin Microbiol*. 2010;48:1782-1786.
16. Espinel-Ingroff A, Arendrup MC, Pfaller MA, et al. Interlaboratory variability of Caspofungin MICs for *Candida* spp. Using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? *Antimicrob Agents Chemother*. 2013;57:5836-5842.

How to cite this article: Oz Y, Gokbolat E. Evaluation of direct antifungal susceptibility testing methods of *Candida* spp. from positive blood culture bottles. *J Clin Lab Anal*. 2018;32:e22297. https://doi.org/10.1002/jcla.22297