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Correlation between E-Test, Disk Diffusion, and Microdilution Methods for Antifungal Susceptibility Testing of Fluconazole and Voriconazole

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The activities of fluconazole and voriconazole against isolates of *Candida* spp. ($n = 400$) were tested by the E-test, disk diffusion, and the National Committee for Clinical Laboratory Standards (NCCLS) M27-A2 broth microdilution-based reference methods. More than 96% of isolates found to be susceptible to fluconazole by the reference method were identified as susceptible by the agar-based methods. Lesser degrees of correlation with the reference method were seen for isolates identified as resistant by the agar-based methods. Interpretive categories are not available for voriconazole, but results qualitatively similar to those for fluconazole were seen. The agar-based E-test and disk diffusion methods are reliable alternatives to the NCCLS M27-A2 reference microdilution method for isolates that test susceptible to fluconazole.

The development of standardized antifungal susceptibility testing methods has been the subject of numerous studies during the last decade. Reference methods for yeasts (the National Committee for Clinical Laboratory Standards [NCCLS] M27-A2 method) and molds (the NCCLS M38-A method) are now available (16). Agar-based susceptibility testing methods have been a focus of interest for many researchers and include the classical disk diffusion (DD) methods and the E-test (ET) method (3, 6–10, 13, 14, 16–18). Those tests are very attractive due to their simplicity, reproducibility, and lack of requirements for specialized equipment (11, 16). Recent studies have documented comparable results between those methods and the results of standard reference broth microdilution (MD) susceptibility testing (7, 11, 13).

In this study, we compared the NCCLS M27-A2 MD method with the ET and DD methods for determination of the susceptibilities of 400 *Candida* species isolates to fluconazole and voriconazole. The ET and DD methods are well studied for fluconazole (3, 6–11, 14), and this work extends their usage to include voriconazole.

MATERIALS AND METHODS

Isolates. Four hundred bloodstream isolates of *Candida* species were randomly selected for testing. These included 205 isolates of *Candida albicans*, 56 isolates of *C. tropicalis*, 39 isolates of *C. glabrata*, 66 isolates of *C. parapsilosis*, 24 isolates of *C. krusei*, and 10 isolates of other species. The isolates were identified with the API 20C AUX system (Biomerieux Vitek, Hazelwood, Mo.) and were subsequently stored in sterile distilled water at room temperature until susceptibility tests were performed. Each isolate was subcultured at least twice on Sabouraud dextrose agar and incubated at 35°C prior to testing to ensure purity and optimal growth.

Inoculum suspensions. Yeast inoculum suspensions were prepared as described for the NCCLS M27-A2 method (12). The turbidity was measured with a spectrophotometer at 530 nm and was adjusted to match a 0.5 McFarland density standard, resulting in a concentration of 1×10^6 to 5×10^6 yeast cells/ml. This inoculum was used directly for inoculation of agar plates (see below) or was diluted as needed for the MD procedure.

Antifungal agents. Antifungal research powders were supplied by Pfizer Inc. (Pfizer Pharmaceuticals Group, New York, N.Y.) and stored at −20°C until they were used. ET strips were obtained from AB Biodisk (Solna, Sweden), with the drug concentrations ranging from 0.016 to 256 μg/ml for fluconazole and 0.002 to 32 μg/ml for voriconazole. Paper disks containing 1 μg of voriconazole were manufactured by Remel, Inc. (Lanexa, Kans.). Paper disks containing 25 μg of fluconazole were manufactured by Becton Dickinson Microbiology Systems (Cockeysville, Md.).

Media and susceptibility testing methods. Broth MD testing was done by the NCCLS M27-A2 MD method and was performed in RPMI 1640 buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid obtained from Sigma Chemical Co. (St. Louis, Mo.). The antifungal agents were tested over final concentration ranges of 0.125 to 64 μg/ml for fluconazole and 0.015 to 16 μg/ml for voriconazole. The plates were incubated at 35°C and read with a spectrophotometer at 570 nm after 24 and 48 h. The MIC was defined as the lowest drug concentration that reduced growth by 50% compared with the growth of the drug-free controls.

The ET and DD methods were performed on Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg of methylene blue (MB; Harleco, Gibbstown, N.J.) per ml due to the ability of that medium to produce enhanced definition of growth margins (5). To prepare the medium, stock solutions of MB (5 mg/ml) and glucose (0.4 g/ml) were made in distilled water. A total of 100 μl of stock MB was added to 100 ml of stock glucose solution to make a stock solution of 0.4 g of glucose per ml plus 5 μg of MB per ml (GMB). The GMB stock solution was filter sterilized and stored at 4°C. Mueller-Hinton agar plates (diameter, 15 cm, with 60 ml of agar; Becton Dickinson Microbiology Systems) were prepared by pouring 2.9 ml of the GMB stock solution on the plate and allowing it to absorb for 4 to 6 h before inoculation.

The agar plates were inoculated by dipping a sterile cotton swab into the inoculum and evenly streaking the swab in three directions over the entire surface of the plate. The plates were allowed to dry for at least 15 min before the ET strips and the disks were applied to the surface. The ET strips and disks with fluconazole and voriconazole were applied onto each inoculated plate, and the plates were incubated at 35°C, with readings taken after 24 and 48 h. Inhibitory zone diameters for the disks and the MICs for the ET strips were measured at the transitional point where growth abruptly decreased, as determined by a marked reduction in colony size, number, and density.

Interpretive breakpoints for fluconazole for the ET and the M27-A2 MD methods follow those published as part of the M27-A2 method: susceptible, ≤8 μg/ml; susceptible-dose dependent, 16 to 32 μg/ml; and resistant, ≥64 μg/ml (12). For the DD method, zone diameters were interpreted on the basis of the

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TABLE 1. Susceptibilities of 400 *Candida* spp. to fluconazole and voriconazole as determined by three methods at two incubation times

Species (no. of isolates tested)	Time (h)	Method	Fluconazole[a]			Voriconazole[a]		
			Range	50%	90%	Range[a]	50%	90%
C. albicans (205)	24	MD	0.125–128	0.125	0.25	0.031–16	0.031	0.031
		ET	0.125–24	1.5	2	0.008–0.750	0.032	0.047
		DD	45–21	33	29	45–25	34	30
	48	MD	0.125–128	0.25	0.5	0.031–32	0.031	0.06
		ET	0.094–>256	1.5	3	0.008–64	0.032	0.064
		DD	47–NZ	33	29	45–15	34	30
C. parapsilosis (66)	24	MD	0.125–64	0.25	1	0.031–2	0.031	0.125
		ET	0.38–>256	1.5	6	0.008–2	0.032	0.094
		DD	43–NZ	36	26	45–12	39	31
	48	MD	0.250–128	0.5	4	0.031–4	0.031	0.25
		ET	0.38–>256	2	16	0.008–64	0.032	0.25
		DD	44–NZ	36	22	45–NZ	38	25
C. tropicalis (56)	24	MD	0.125–128	0.25	1	0.031–32	0.031	0.125
		ET	0.380–>256	1.5	4	0.016–1.5	0.064	0.19
		DD	40–10	33	28	40–12	30	25
	48	MD	0.125–128	1	16	0.031–32	0.125	1
		ET	0.750–512	2	6	0.019–64	0.19	0.75
		DD	38–NZ	28	24	37–NZ	24	18
C. glabrata (39)	24	MD	0.250–64	4	8	0.031–2	0.125	1
		ET	2–512	8	32	0.047–2	0.125	0.38
		DD	38–NZ	27	20	35–12	29	24
	48	MD	0.250–128	8	32	0.031–8	0.5	2
		ET	4–512	24	64	0.094–3	0.38	1
		DD	32–NZ	19	12	32–NZ	20	15
C. krusei (24)	24	MD	0.5–128	16	16	0.031–1	0.125	0.25
		ET	1.5–>256	48	96	0.016–105	0.19	0.38
		DD	38–NZ	18	14	42–12	28	23
	48	MD	1–128	32	64	0.031–2	0.5	0.5
		ET	2–512	512	512	0.016–12	0.75	2
		DD	38–NZ	0	0	42–NZ	16	13
Other species (10)	24	MD	0.125–2	0.25	0.5	0.031	0.031	0.031
		ET	0.5–2	0.75	1	0.006–0.023	0.016	0.016
		DD	45–36	40	38	44–38	41	38
	48	MD	0.125–4	0.5	1	0.031–0.125	0.031	0.031
		ET	0.5–4	1	1	0.006–0.023	0.016	0.016
		DD	45–36	42	40	45–38	43	40

[a] The values are MICs (in micrograms per milliliter) for the MD and ET methods and inhibition zone diameter (in millimeters) for the DD method. The value shown is the lowest MIC that was greater than (for the MD and ET methods) or the greatest zone diameter that was less than (for the DD method) 50 or 90% of the observed values, as indicated. NZ, no zone.

work of Barry et al. (5), with zone diameters of ≥19 mm indicating susceptibility, zone diameters of 15 to 18 mm indicating susceptible-dose dependent, and zone diameters of ≤14 mm indicating resistance.

Quality control isolates *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included in all runs, and all results were within published limits (4, 12).

RESULTS AND DISCUSSION

Table 1 shows the drug MICs and zone diameters obtained by all three methods after both 24 and 48 h of incubation. Overall, the ET method tended to give slightly higher fluconazole MICs than the MD method at both time points, whereas the voriconazole MICs by the ET and MD methods were similar at both time points.

As shown in Table 2, the overall levels of agreement between the MICs obtained by the MD and ET methods at 24 and 48 h were good for both fluconazole and voriconazole. For both drugs, the agreement between the ET MIC at 48 h and

TABLE 2. Percentage of paired MD and ET MICs within 2 doubling dilutions

Time (h) of ET reading	Correlation (%) of ET and MD MICs			
	Fluconazole		Voriconazole	
	24 h	48 h	24 h	48 h
24	99.5	96.5	98.0	87.0
48	99.8	97.0	98.8	93.3

the reference (MD) MIC at 48 h was >93%. However, better correlations were noted for the readings obtained at 24 h, when the percent agreement was >98% for the two drugs. Disparate readings were generally attributable to trailing growth: for isolates for which there was a difference, the ET MICs tended to be lower at both time points and to be lower by the MD method at 24 h, but the MD MICs tended to be elevated at 48 h.

Comparisons of the results obtained by all three methods are shown in Table 3 by interpretive category for fluconazole. Isolates that tested susceptible by the DD or ET method at either time point had a ≥96% likelihood of testing susceptible or susceptible-dose dependent by the MD method. The MICs for isolates testing resistant by the DD and ET method were almost always in the susceptible-dose dependent or resistant category by the MD method, whereas isolates testing resistant by the MD method produced results by the agar-based method ranging from susceptible to resistant. Thus, the correlation between the resistant categories for the MD method and the agar-based methods was ≤83%.

Table 4 correlates the voriconazole MICs obtained by the MD method and the inhibition zone diameters obtained by the DD method after 24 and 48 h of incubation. The best correlation was obtained with readings obtained at 24 h for both methods. After 48 h, trailing growth similar to that noted above for fluconazole tended to generate higher MICs.

As noted by others (5, 11), use of Mueller-Hinton agar flooded with GMB enhanced growth and simplified reading relative to the MD method. In addition, the trailing phenomenon was less pronounced by both agar-based methods. As others have shown that performing the ET method on RPMI 1640 or Casitone (the medium suggested by the manufacturer) produces results comparable to those obtained by the reference MD method (5), we did not repeat that work. Rather, we tested the isolates by the ET method on GMB-supplemented Mueller-Hinton agar. The results obtained by both the DD and the ET methods were in acceptable concordance with those obtained by the MD method, with the exception of the recurring problem of discrepancies due to isolates that showed trailing growth.

In summary, the agar-based ET and DD methods are reliable alternatives to the NCCLS M27-A2 reference MD method for isolates that test susceptible to fluconazole. However, the detection of resistance by agar-based methods correlates poorly with the detection of resistance by the reference NCCLS M27-A2 method. Specifically, ≥90% of the isolates that tested resistant to fluconazole by an agar-based method tested susceptible-dose dependent or resistant by the reference MD method. Conversely, 30 to 50% of the isolates that tested resistant by the MD method appeared to be susceptible when they were tested by agar-based methods. This difference was principally due to trailing growth associated with the MD method. Prior work suggests that the results for isolates with significant trailing should be interpreted on the basis of the lower MIC observed at the earlier time point (1, 2, 12, 15). Our data thus suggest that the results for isolates that appear to be resistant by any method should be carefully reviewed and that such isolates may merit repeat testing and/or testing by an

TABLE 3. Comparison of interpretive categories for fluconazole and rates of interpretive agreement[a]

Method	Incubation time (h)	Category	Fluconazole MIC50 (μg/ml) at 48 h by MD			Predictive values (%)	No. (%) of discrepant results[b]			No. (%) of total agreement
			S	S-DD	R		Minor	Major	Very major	
DD	24	S	337	23	15	96.0[c]	30 (7.5)	0 (0.0)	15 (3.8)	355 (88.8)
		I	1	8	4					
		R	0	2	10	83.0[d]				
	48	S	329	5	10	97.1[c]	27 (6.7)	4 (1.0)	10 (2.5)	359 (89.8)
		I	5	11	0					
		R	4	17	19	47.5[d]				
ET	24	S	334	14	11	97.0[c]	32 (8.0)	1 (0.3)	11 (2.8)	356 (89.0)
		I	3	9	5					
		R	1	10	13	54.2[d]				
	48	S	319	0	9	97.3[c]	43 (10.7)	5 (1.3)	9 (2.3)	343 (85.8)
		I	14	5	1					
		R	5	28	19	36.5[d]				

[a] S, susceptible; I, intermediate; R, resistant; S-DD, susceptible-dose dependent; MIC_{50}, MIC at which 50% of isolates are inhibited.
[b] Minor discrepancies, susceptible-dose dependant by one method but susceptible or resistant by the other; Major discrepancies, resistant by the test method but susceptible by the reference test; very major discrepancies, susceptible by the test method but resistant by the reference test.
[c] The value shown is the percentage of the time that a result of susceptible by the agar-based method correlated with a susceptible or susceptible-dose dependent result by the reference MD method.
[d] The value shown is the percentage of the time that a result of resistant by the agar-based method correlated with a result of resistance by the reference MD method.

TABLE 4. Correlation between results of MD and DD methods for voriconazole after 24 and 48 h of incubation

Time and disk zone diam (mm)	No. of isolates for which the MD MIC (μg/ml) at the indicated times was as follows:																	
	24 h MD MICs									48 h MD MICs								
	0.031	0.062	0.125	0.250	0.5	1.0	2.0	>4.0	Total	0.031	0.062	0.125	0.250	0.5	1.0	2.0	>4.0	Total
24 h																		
45	3								3	3								3
44	8								8	8								8
43	6								6	6								6
42	11								11	10	1							11
41	8		1					1	10	9					1			10
40	17								17	14	1		1	1				17
39	14		1						15	13	1			1				15
38	24								24	17	3	1	3					24
37	15	1							16	11	3	1					1	16
36	22								22	21							1	22
35	38	1							39	30	6	1		1	1			39
34	37								37	33	2				1		1	37
33	27	3							30	19	3	1	5	1			1	30
32	33	2						1	36	27	3	1	1	1		1	2	36
31	13	2	4	1					20	9	3	1	3	3		1		20
30	17	3	4	2		1			27	12	1	2	2	6	3		1	27
29	6	2	4						12	4	2	1	1	3	1			12
28	7	7	2	2					18	2	3	3	1	5	3		1	18
27	4	1	4	2	1				12		3	2		4	1	2		12
26	1	1	4					1	7		2			2	1		2	7
25	1	2	4	3		1		1	12		1		3	3	2	1	2	12
24			1						1					1				1
23		2	1	1					4		1	1		1		1		4
22		1	1	1					3			1		2				3
21			1	1					1					1				1
20				1					1					1				1
18							1		1							1		1
16				1					1							1		1
12					2	3	1		6						1	4	1	6
Total for 24 h	312	28	31	15	3	5	2	4	400	248	39	16	20	37	15	12	13	400
48 h																		
45	7								7	7								7
44	10								10	9	1							10
43	14								14	14								14
42	5								5	5								5
41	5								5	5								5
40	11		1					1	13	11			1		1			13
39	10								10	9	1							10
38	15		1						16	13	1	1			1			16
37	12								12	10	1						1	12
36	24								24	22		1			1			24
35	26								26	21	3			2				26
34	39								39	37	1						1	39
33	21								21	18	2						1	21
32	29	2							31	25	5	1						31
31	6								6	5	1							6
30	24								24	20	2			1	1			24
29	8		1						9	4	3		1				1	9
28	10	4							14	4	3	1	2	2			2	14
27	5	1							6	1	2	1		1			1	6
26	4	1							5	2	2				1			5
25	6	1	1	1				1	9	3	1	1	3			1		9
24	2	3	1					2	8	1		1	1	2			3	8
23	5	2							7		2	2	3					7
22	6	3	1						10	1	1	1	1	3	1		2	10
21	3	3							6		4	1				1		6
20	4	3	6						13	1	2	2	1	3	4			13
19			1	1					2					1	1			2
18	1	4	5	1	1	1			13		1	3	3	5		1		13
17		1	1	2					4					3	1			4
16			4	6					10				1	5	2	2		10
15			5			1			6					4	2			6
14			1	2					3					3				3
13			1	2			1		4				1	1		2		4
0			1	1	2	3	1		8					1	1	5	1	8
Total for 48 h	312	28	31	15	3	5	2	4	400	248	39	16	20	37	15	12	13	400

alternative method. Although more work needs to be done with less susceptible isolates, the aggregate data suggest that agar-based methods appear to produce a more consistent in vitro-in vivo correlation than the reference MD method by eliminating trailing growth from the equation. The lack of interpretive breakpoints for voriconazole makes such comparisons impossible for this newer triazole; however, analysis of numeric MICs and the corresponding zone diameters for this compound suggests conclusions similar to those for fluconazole.

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