JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1996, p. 842–847
0095-1137/96/$04.00+0

Vol. 34, No. 4

Fluconazole and Amphotericin B Antifungal Susceptibility Testing by National Committee for Clinical Laboratory Standards Broth Macrodilution Method Compared with E-test and Semiautomated Broth Microdilution Test

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Received 2 August 1995/Returned for modification 10 October 1995/Accepted 18 January 1996

A comparative study of fluconazole and amphotericin B susceptibility testing was performed with 68 clinical *Candida* species isolates and three test methods. The methods used were an agar diffusion method (E-test) and two broth dilution methods, the National Committee for Clinical Laboratory Standards (NCCLS) reference broth macrodilution method and an in-house-prepared semiautomated broth microdilution method based on the Bioscreen turbidometer. In the microdilution method, growth of the yeasts was measured continuously by the automatic turbidometer (Bioscreen), which permitted precise and objective determination of endpoints. MIC endpoints were read after 24 h for the microdilution method and the E-test. Amphotericin B susceptibility testing with the NCCLS method and the E-test yielded comparable results in 89% of the tests, meaning that the endpoints obtained were identical or differed by no more than 2 twofold dilutions. The NCCLS and broth microdilution tests scored 97% comparable results, and the E-test and the broth microdilution test yielded 90% comparable results. Fluconazole susceptibility testing produced 96% comparable results with the NCCLS test and the E-test, 100% comparable results with the NCCLS and the microdilution methods, and 98.5% comparable results with the microdilution method and the E-test. We conclude that the E-test and the Bioscreen microdilution method are valuable alternatives to the NCCLS reference method for routine susceptibility testing of *Candida* species with fluconazole and amphotericin B.

All over the world, invasive fungal infections are steadily increasing in frequency and clinical importance. This trend has been attributed to the growing group of immunocompromised patients (1), for whom fungal infections have become a major cause of morbidity and mortality in the last few years (2). Even more disturbing is the growing number of reports on resistance of fungi against commonly used antifungal drugs (10). Consequently, there is a need for a precise, clinically relevant, easy-to-perform in vitro susceptibility testing method to guide antifungal therapy and monitor local resistance patterns (14). However, unlike bacteriological susceptibility testing, fungal susceptibility testing is still in its infancy. Nevertheless, considerable progress has been made, particularly by standardization of test conditions to prevent intra- and interlaboratory discrepancies in antifungal susceptibility testing (6, 14).

The 1992 National Committee for Clinical Laboratory Standards (NCCLS) guidelines for susceptibility testing of *Candida* species and *Cryptococcus neoformans* described a broth macrodilution method using semisynthetic RPMI 1640 medium at pH 7.0, with a spectrophotometrically standardized inoculum and fixed incubation and endpoint reading criteria (8). The NCCLS method, however, is labor-intensive and time-consuming and therefore less suited for routine fungal susceptibility testing.

The recently introduced E-test (AB Biodisk, Solna, Sweden) consists of a strip impregnated with an antifungal agent that is applied to an inoculated agar plate in order to create a predefined antifungal gradient in the agar. Although the E-test has been widely and successfully used in bacterial susceptibility testing, experience with this method in routine susceptibility testing of fungi is limited.

The broth microdilution method that we describe in this report follows most of the guidelines prescribed in the NCCLS document. It makes use of a computer-controlled dispenser-shaker-incubator-turbidometric reader (Bioscreen; Labsystems, Helsinki, Finland), which reduces the preparation time and culture medium volume needed. The Bioscreen system also permits continuous measurement of the growth curves of the fungi. This system combines ease of use with flexibility and may offer interesting information on the kinetics of fungal growth in the presence of antifungal agents.

In this study, we compared the NCCLS macrodilution method with the E-test and with the new broth microdilution method for the determination of MIC endpoints for amphotericin B and fluconazole against *Candida* species. We evaluated the E-test and the broth microdilution methods as possible alternatives for routine fungal susceptibility testing.

MATERIALS AND METHODS

Organisms. Sixty-eight clinical yeast isolates from deep samples and blood cultures and six reference strains with known susceptibility patterns (*Candida parapsilosis* ATCC 22019 and 90018, *Candida albicans* ATCC 90028 and 90029, *Candida glabrata* ATCC 90030, and *Candida krusei* ATCC 6258) were selected for comparative susceptibility testing with amphotericin B and fluconazole. The clinical isolates included *C. glabrata* ($n = 29$), *C. albicans* ($n = 20$), *C. parapsilosis* ($n = 7$), *C. krusei* ($n = 7$), *Candida lusitaniae* ($n = 2$), *Candida tropicalis* ($n = 1$), *Candida rugosa* ($n = 1$), and *Candida guilliermondii* ($n = 1$). These strains were identified with the API 20C system (bioMérieux, Marcy l'Etoile, France). If needed, microscopical examination of morphology on cornmeal agar (Oxoid) was used to confirm the identity.

For short-term storage, isolates were grown on Sabouraud agar slants (Sanofi

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TABLE 1. Comparative susceptibility testing results for 68 clinical isolates and 4 reference strains for fluconazole and amphotericin B

Species	Isolate	MIC endpoint (μg/ml)					
		Fluconazole			Amphotericin B		
		E-test	NCCLS	Bioscreen	E-test	NCCLS	Bioscreen
C. glabrata	15	2	4	8	0.12	0.25	0.25
	18	>16	8	4	0.12	0.5	0.5
	19	8	8	8	1	1	0.5
	20	0.12	0.5	0.25	0.25	1	0.5
	21	>16	>16	>16	0.12	0.25	0.25
	24	8	8	16	0.25	0.5	0.5
	37	>16	>16	>16	0.03	0.12	0.06
	44	4	16	8	0.06	0.25	0.25
	48	2	8	4	0.06	1	0.25
	51	4	16	8	0.12	1	0.5
	52	8	>16	8	2	1	1
	56	>16	16	8	1	1	0.5
	58	16	8	8	0.25	0.5	0.5
	59	16	>16	8	0.5	0.12	0.5
	75	1	4	4	0.25	0.5	0.5
	76	1	8	4	0.5	1	0.5
	77	2	8	4	0.12	0.5	0.25
	78	8	>16	>16	0.25	1	0.5
	80	2	>16	8	0.25	0.5	1
	84	2	8	4	0.25	1	0.5
	85	8	16	4	0.5	1	0.5
	88	>16	8	16	0.12	0.12	0.25
	90	>16	8	16	0.12	0.25	0.5
	91	0.5	2	1	0.25	1	0.5
	92	16	16	8	0.25	0.5	0.5
	94	4	8	4	0.25	1	0.5
	101	8	8	8	0.25	1	0.5
	107	8	4	4	0.25	1	0.5
	108	8	8	4	0.25	1	0.5
	85	8	8	16	0.25	1	0.5
C. albicans	28	>16	>16	8	0.12	0.12	0.25
	42	>16	16	>16	0.12	0.12	0.25
	43	>16	16	8	0.25	0.12	0.25
	46	0.5	0.25	0.25	0.25	0.5	0.12
	47	0.12	0.25	0.25	0.12	0.25	0.12
	53	0.5	0.5	0.25	0.25	0.5	0.25
	60	0.12	0.12	0.25	0.06	0.12	0.12
	61	0.25	0.25	0.25	0.03	1	0.25
	68	0.06	0.25	0.25	<0.03	0.25	0.12
	69	0.12	0.25	0.12	0.06	<0.03	0.25
	73	0.5	0.5	0.12	0.12	0.25	0.5
	81	2	4	2	0.12	0.5	0.5
	82	0.06	0.12	0.12	0.12	0.5	0.25
	95	2	2	4	0.03	0.06	0.25
	96	0.5	1	1	0.12	0.25	0.25
	97	0.12	0.25	0.25	0.12	0.25	0.25
	100	0.12	0.5	0.25	0.5	0.25	0.5
	109	0.25	1	0.25	0.25	0.5	0.5
	110	0.12	0.12	0.25	0.03	0.25	0.06
	134	>16	>16	>16	0.12	0.5	0.5
	46	0.5	0.5	0.5	0.12	0.5	0.12
	60	0.25	0.25	0.5	0.12	0.12	0.12
C. parapsilosis	22	0.5	2	1	0.25	0.12	0.25
	49	0.25	1	0.5	<0.03	0.06	0.25
	50	2	8	4	<0.03	0.5	0.25
	55	0.25	0.5	0.25	0.06	0.12	0.12
	57	0.12	0.25	0.25	<0.03	0.25	0.06
	70	0.25	2	0.5	0.03	0.25	0.25
	83	0.5	1	0.5	0.5	0.12	0.5
	55	0.12	0.25	0.5	0.12	0.12	0.5
C. krusei	40	>16	>16	>16	0.5	1	1
	54	>16	>16	>16	2	0.5	0.5

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TABLE 1—*Continued*

Species	Isolate	MIC endpoint (μg/ml)					
		Fluconazole			Amphotericin B		
		E-test	NCCLS	Bioscreen	E-test	NCCLS	Bioscreen
	62	>16	>16	>16	2	0.5	1
	63	>16	>16	>16	2	0.5	1
	64	>16	>16	>16	0.25	0.5	0.5
	74	>16	>16	>16	1	1	1
	98	>16	>16	>16	1	1	0.5
C. lusitaniae	65	0.5	0.5	0.5	<0.03	0.12	0.25
	93	0.25	1	0.5	<0.03	0.12	0.25
C. tropicalis	23	0.25	0.12	0.25	0.12	0.06	0.06
C. rugosa	26	4	8	8	0.5	1	0.12
C. guilliermondii	36	2	4	8	<0.03	<0.03	0.06

Diagnostics, Pasteur) and stored at room temperature. For prolonged storage, a suspension in Sabouraud broth with glycerol (9:1 [vol/vol]) was frozen at −70°C.

Inoculum. Prior to testing, each isolate was grown on Sabouraud dextrose agar (Sanofi Diagnostics, Pasteur) for 24 h at 36°C. Suspensions were prepared from individual colonies (diameter, ≥1 mm) in 5 ml of sterile 0.85% saline to a density of a 0.5 McFarland standard.

Antifungal agents. Amphotericin B was purchased from Bristol-Myers Squibb (Brussells, Belgium) as a lyophilized powder for intravenous administration (Fungizone). Fluconazole was supplied as a solution for intravenous administration (Diflucan) by Pfizer (Brussels, Belgium). Amphotericin B was dissolved in dimethyl sulfoxide at a concentration of 1,280 μg/ml, and fluconazole was dissolved in 0.85% saline also at a concentration of 1,280 μg/ml. Stock solutions were stored at −70°C until used.

Assay media. RPMI broth was prepared from RPMI 1640 broth medium (Sigma R7880) supplemented with 0.3 g of glutamine (Janssen, Beerse, Belgium) per liter, buffered with 34.6 g of morpholinepropanesulfonic acid (MOPS) per liter and adjusted to pH 7.0. This medium contains 2 g of glucose per liter and was used for the NCCLS broth macrodilution method and the Bioscreen broth microdilution method.

RPMI agar for the agar diffusion E-test was prepared the same way as the RPMI broth and supplemented with 18 g of glucose per liter and 15 g of Bacto agar per liter according to E-test technical guide 4b (AB Biodisk).

Susceptibility testing methods. (i) NCCLS broth macrodilution reference method. The NCCLS broth macrodilution reference method was performed according to NCCLS document M27-P (8). A working suspension of the inoculum was made by a 1:100 dilution of the 0.5 McFarland standard yeast suspension in 0.85% saline followed by a 1:20 dilution in RPMI broth. Twofold dilutions of the antifungal agents from 128 to 0.03 μg/ml were prepared with the working suspension of the inoculum. The tubes were incubated at 36°C for 48 h. MIC endpoints were defined as the lowest concentration causing growth of ≤20% of the control level for fluconazole and as the lowest concentration causing growth of ≤5% of the control level for amphotericin B.

(ii) Agar diffusion E-test. For the agar diffusion E-test (AB Biodisk), 0.5 McFarland standard inocula were applied to RPMI agar with a cotton swab. The plates were allowed to dry for at least 15 min before the E-test strip was applied to the surface. MIC endpoints were read after 22 to 25 h of incubation at 36°C, according to the manufacturer's instructions (E-test technical guide 4b). For fluconazole, if diffuse growth of microcolonies up to the strip was observed, the MIC endpoint was selected at the point of approximately 80% growth inhibition. For fluconazole, MIC endpoints were read macroscopically; for amphotericin B, a stereoscope was used.

(iii) Bioscreen broth microdilution test. The Bioscreen broth microdilution test relies on a computer-controlled dispenser-shaker-incubator-reader (Bioscreen) which permits automated dilution of antifungal agents and continuous turbidometry of fungal growth. Twofold dilutions (4 to 0.03 μg/ml for amphotericin B and 16 to 0.12 μg/ml for fluconazole) were prepared with the Bioscreen system in RPMI broth starting from two stock solutions for each antifungal drug (16 and 1 μg/ml for amphotericin B and 64 and 4 μg/ml for fluconazole). A working suspension of the inoculum (0.5×10^4 to 2.5×10^4 CFU) was made by a 1:25 dilution of the 0.5 McFarland standard yeast suspension in saline, and 40 μl was added to the microtiter plates with a multichannel pipette to a final volume of 320 μl. With this inoculum, the exponential growth phase started after 8 to 12 h and the stationary phase of growth control cultures was reached after 18 to 20 h of incubation in more than 95% of tests. All tests were performed in duplicate.

Two flat-bottomed 100-well (10 by 10) microtiter plates could be incubated simultaneously. Yeasts were incubated at 36°C for 22 h, and fungal growth was measured at regular programmable intervals (every 10 min). Between turbidity measurements, the microtiter plates were shaken continuously.

The MIC endpoint for fluconazole was defined as the lowest drug concentration for which the following equation is true: $A \leq A_b + [0.2 \times (A_k - A_b)]$, where A is the absorbance, k is a growth control well (no antifungal agent added), and b is a blank control well (no inoculum added). The MIC endpoint for amphotericin B was defined at the lowest drug concentration for which the following equation is true: $A \leq A_b + [0.05 \times (A_k - A_b)]$.

RESULTS

Seventy-two amphotericin B susceptibility tests of clinical isolates, comparing the three methods, were performed (Table 1). In 89% of the tests, the MIC endpoints obtained with the NCCLS method and the E-test were identical or comparable (differing by no more than 2 twofold dilutions). The NCCLS and broth microdilution test results were 97% identical or comparable, and the E-test and broth microdilution test results were 90% comparable. In 80.5% of the tests, the MIC endpoints obtained by all three methods differed by no more than 2 twofold dilutions.

Seventy-two comparative susceptibility tests of clinical isolates were performed with fluconazole (Table 1); for the NCCLS method and the E-test, 96% of the results differed by no more than 2 twofold dilutions; for the NCCLS and the microdilution methods, 100% of the tests were comparable, and for the microdilution method and the E-test, 98.5% of the test results were comparable. In 94.5% of the tests, the three methods gave MIC endpoints that differed by no more than 2 twofold dilutions.

Figure 1 shows the frequency distributions of MIC endpoints obtained by the three methods with amphotericin B. From Fig. 1, it can be deduced that the E-test gave systematically lower MIC endpoints than the NCCLS and microdilution methods. This is also shown in Fig. 2, which shows scattergrams of the MIC endpoints obtained for each isolate with amphotericin B with the NCCLS method and the E-test. On average, the MIC endpoints for amphotericin B obtained with the E-test were 1 twofold dilution lower than those obtained with the microdilution method and 1.5 twofold dilutions lower than those obtained with the NCCLS method.

For fluconazole, the E-test and the microdilution method gave somewhat lower MIC endpoints than the NCCLS method; on average, the E-test gave MIC endpoints 0.6 twofold

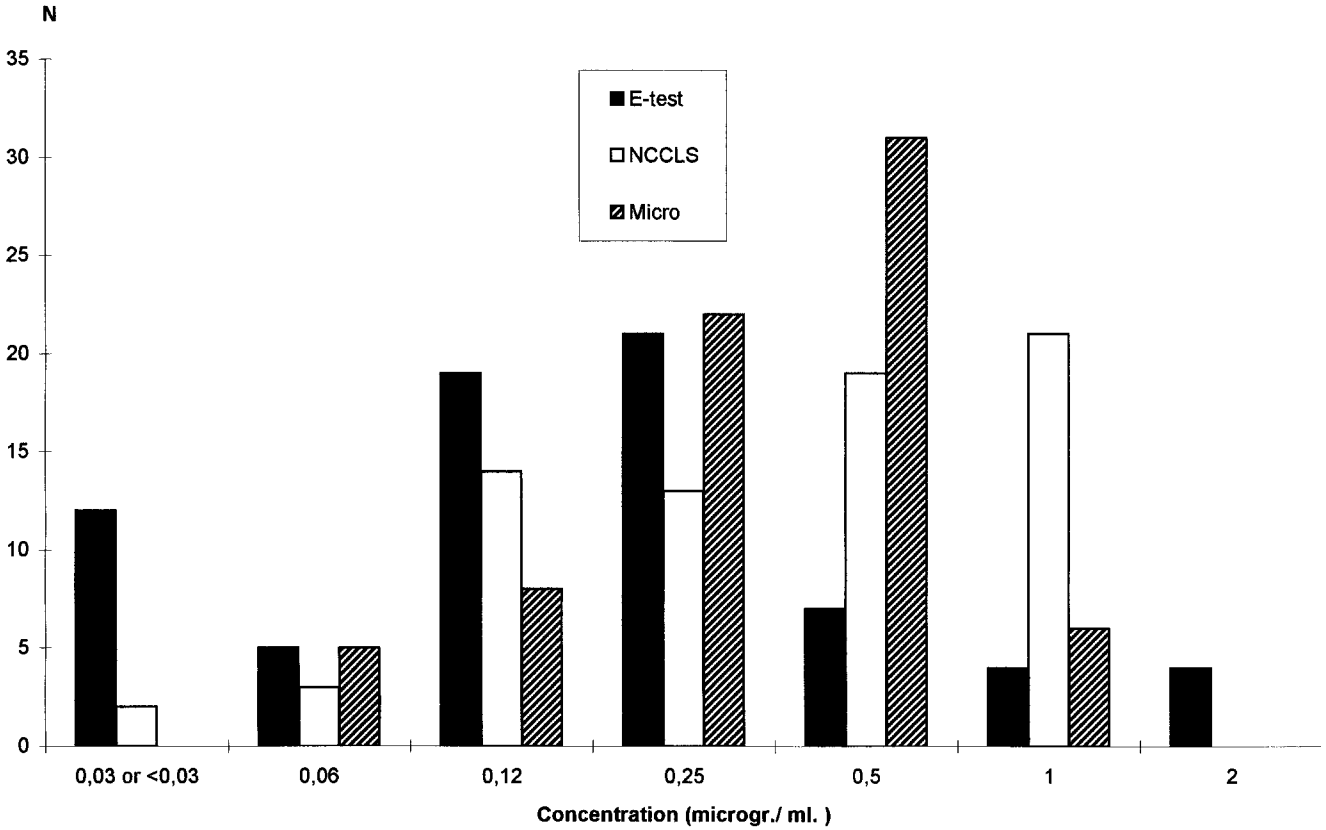

FIG. 1. Frequency distribution of amphotericin B MIC endpoints obtained by each of the three methods.

dilutions lower and the microdilution method gave MIC endpoints 0.4 twofold dilutions lower than those of the NCCLS method. Figure 3 gives the frequency distributions of MIC endpoints obtained by all three methods with fluconazole. The isolates for which the MIC endpoints were higher than 1 μg/ml were predominantly *C. glabrata* and *C. krusei* strains.

Trailing of fungal growth in fluconazole susceptibility testing was encountered with all three methods and was particularly prominent for *C. glabrata* isolates (60% of all isolates). Reading of the MIC endpoints by the microdilution method, however, was not hampered by trailing because turbidometry allowed precise determination of the fluconazole concentration that led to 80% growth inhibition. Addition of 18 g of glucose per liter to the culture medium to facilitate endpoint reading (15) in the microdilution method did not lead to a significant improvement for the majority of tests.

With fluconazole, the E-test often produced inhibition zones with diffuse edges. In 65% of the tests, a double zone with growth of microcolonies throughout the inhibition zone was seen. This pattern was most often seen with *C. albicans*. For 80% of the *C. glabrata* isolates, growth of microcolonies with decreasing intensity just inside the border of the inhibition zone was observed.

With amphotericin B, the E-test produced a long and narrow inhibition zone with sharp endpoints. In 20% of the tests, microcolonies could be found inside the inhibition zone. Because of these microcolonies, endpoint reading was done with a stereoscope. Reproducibility testing of all three methods with six reference strains produced endpoints that differed by no more than 2 twofold dilutions (Table 2) and were within the ranges reported by Pfaller et al. (11).

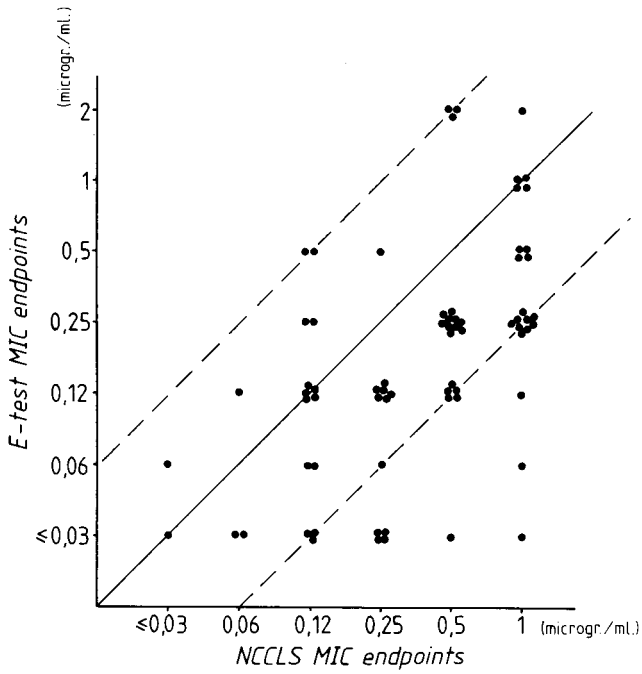

FIG. 2. Scattergram of amphotericin B MIC endpoints obtained with the E-test versus the NCCLS reference method.

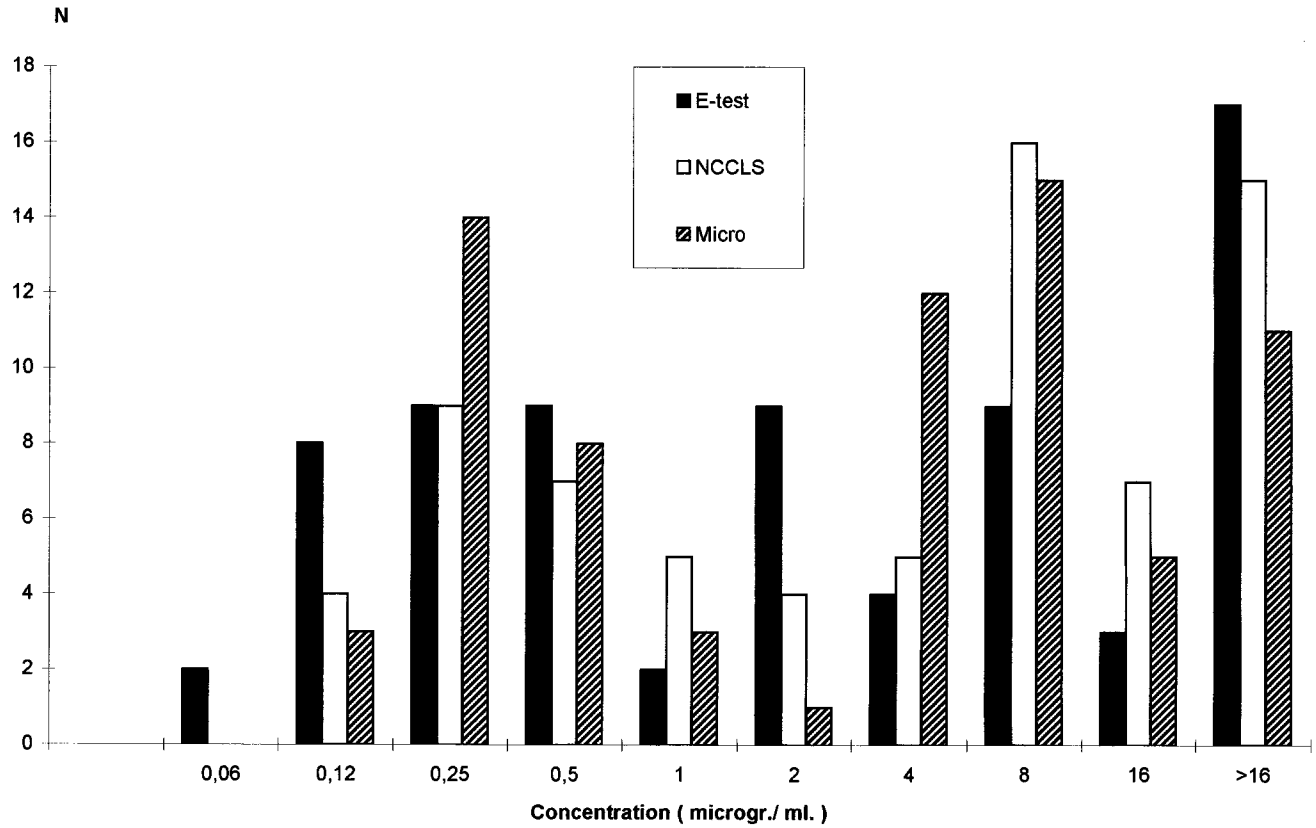

FIG. 3. Frequency distribution of fluconazole MIC endpoints obtained by each of the three methods.

DISCUSSION

Our results show that both the E-test and the Bioscreen broth microdilution method are promising alternatives to the NCCLS reference broth macrodilution method for susceptibility testing of yeasts with fluconazole and amphotericin B.

Earlier studies had already shown a good agreement between microdilution methods and the NCCLS macrodilution method for fluconazole and amphotericin B susceptibility testing (3, 12, 15). A recent study comparing the NCCLS reference macrodilution method with a microdilution method and the E-test also found excellent agreement between these tests for fluconazole susceptibility testing (16).

The agar dilution-based E-test has the advantage of being simple and easy to use. Agar-based techniques have been used extensively for susceptibility testing of yeasts because they are easy to perform and economical and do not require expensive instruments. Their reproducibility, however, depends on good standardization of the inoculum, the temperature, and the incubation time (14).

Endpoint reading for the E-test was done after 24 h. Although a shorter incubation time has been shown to lead to more variability in MIC endpoints, we found that endpoint reading after 48 h for the E-test led to a lower correlation between E-test MIC endpoints and those obtained with the NCCLS method (data not shown). In accordance with the observation of Sewell et al. (16), we also noticed that E-test MIC endpoints for *C. glabrata* strains tended to be lower than those obtained with the NCCLS method after 48 h. However, we did not find the overall poor correlation (37%) reported by Sewell et al. (16) between MIC endpoints for fluconazole obtained by the E-test and the NCCLS method for *C. glabrata* (93% correlation for our isolates). As previously reported (4, 9), the absolute endpoints obtained for fluconazole with the E-test tended to be lower than those obtained with the broth macrodilution method but not lower than those obtained with the broth microdilution method.

E-test MIC endpoints for amphotericin B were generally lower than those obtained with the NCCLS and microdilution methods. This compression of the amphotericin B MIC range in RPMI 1640 medium has been reported by others, too (7, 14). Preliminary testing of a proven amphotericin B-resistant *C. lusitaniae* strain produced MIC endpoints of 1.5 μg/ml for the E-test, 2 μg/ml for the NCCLS method, and 1 μg/ml for Bioscreen (unpublished results). These results tend to confirm the reported inadequacy of RPMI 1640 medium for distinguishing between amphotericin B-resistant and -susceptible isolates (13, 14).

The main problem associated with the agar diffusion E-test was the difficulty in defining the precise borders of the inhibition zone, particularly when fluconazole was tested. To prevent variability in endpoint reading, plates were always scored by the same person. In a routine situation, however, trailing might constitute an important source of endpoint variability (5), and extensive training combined with the use of controls illustrating various growth patterns may be required.

The Bioscreen broth microdilution method combines the characteristics of a microdilution system with the advantages of partial automation and continuous turbidometry. The hands-

TABLE 2. Reproducibility of susceptibility testing with six reference strains

Strain	MIC endpoint (μg/ml)					
	Fluconazole			Amphotericin B		
	E-test	NCCLS	Bioscreen	E-test	NCCLS	Bioscreen
C. parapsilosis ATCC 90018	0.12	0.5	0.5	0.5	0.5	0.5
	0.12	0.25	0.5	0.5	0.25	0.5
	0.25	0.5	0.5	0.25	0.25	0.25
	0.5	0.5	0.5	0.25	0.5	0.12
C. albicans ATCC 90028	0.5	0.5	0.5	0.25	0.5	0.12
	0.5	0.5	0.5	0.12	0.5	0.5
	0.5	1	0.5	0.25	0.5	0.5
	0.5	0.25	0.25	0.12	0.25	0.25
C. albicans ATCC 90029	0.5	0.25	0.5	0.25	0.12	0.5
	0.25	0.25	0.25	0.12	0.25	0.12
	0.5	0.25	0.25	0.25	0.5	0.25
	0.12	0.25	0.25	0.12	0.5	0.25
C. parapsilosis ATCC 22019	2	2	4	0.5	0.5	0.5
	2	1	4	0.5	1	0.5
	2	4	2	0.25	0.5	0.25
	2	2	2	0.25	0.25	0.12
C. krusei ATCC 6258	64	32	32	0.75	0.5	0.5
	64	16	32	0.75	0.5	0.5
	32	16	32	1	1	1
	32	32	16	1	1	1
C. glabrata ATCC 90030	32	8	16	0.25	0.5	0.5
	32	4	16	0.5	0.25	0.5
	8	4	8	0.12	0.5	0.5
	8	4	8	0.25	0.5	0.25

on time for testing four isolates with two antifungal agents was around 50 min. The incubation time and temperature, shaking interval, and intervals between turbidometric readings of fungal growth were all programmed and executed automatically. We found that microtiter plates had to be shaken continuously during the incubation period to prevent yeasts from sedimenting to the bottom of the cup. Because turbidometry allowed objective and precise determination of the endpoint relative to the growth in the control well, standardization of the inoculum was less critical than in the macrodilution method. This would make it possible to use larger inocula for slowly growing strains. For most strains for which the MIC endpoints were high, we found a gradual decline of the height of the stationary-phase turbidity with increasing concentrations of the antifungal agent. Contrary to the observations of Rodriguez-Tudela and Martinez-Suarez (15), addition of glucose in most cases did not lead to a better distinction between less than and more than 80% growth inhibition. Overall, the endpoints obtained with the Bioscreen microdilution method correlated better than the E-test with the NCCLS values. The Bioscreen microdilution endpoints, however, also correlated better with the E-test values than the NCCLS endpoints correlated with those of the E-test.

We conclude that the E-test and the Bioscreen microdilution method are valuable alternatives to the NCCLS reference method for routine susceptibility testing of *Candida* species with fluconazole and amphotericin B. The E-test is particularly user-friendly. The Bioscreen microdilution method offers the advantages of automation, objective and precise endpoint determination, and the possibility of studying the growth kinetics of yeasts in the presence of antifungal agents.

ACKNOWLEDGMENT

We thank F. Odds (Janssen) for providing us with an amphotericin B-resistant *C. lusitaniae* strain.

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