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Susceptibility Testing of Fluconazole by the NCCLS Broth Macrodilution Method, E-Test, and Disk Diffusion for Application in the Routine Laboratory

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Antifungal susceptibility testing may be an important aid in the treatment of patients with life-threatening yeast infections. In order to establish the suitability of different susceptibility test methods for fluconazole with yeasts, the Rosco tablet and the E-test were compared with the gold standard NCCLS broth macrodilution method for 106 yeast strains. These included 102 clinical isolates of *Candida* spp., including *Candida glabrata* ($n = 30$), *Candida albicans* ($n = 20$), *Candida tropicalis* ($n = 13$), *Candida parapsilosis* ($n = 10$), *Candida krusei* ($n = 8$), plus *Cryptococcus neoformans* ($n = 3$), *Saccharomyces cerevisiae* ($n = 2$), and 16 strains belonging to other *Candida* spp. Four American Type Culture Collection strains of *Candida* were included as quality controls. The NCCLS method was found to be too complex and labor-intensive for routine testing. The E-test is an accurate alternative, but experience in determining MICs and careful attention to procedural details are critically important. The Rosco tablet showed the best agreement with the NCCLS reference method, especially when newly established breakpoints of $R \leq 10$ mm and $S \geq 21$ mm were used.

During the last decade, the higher incidence of fungal infections in hospitalized patients has resulted in the use of systemic antifungal agents, especially fluconazole, which remains a first-line antifungal agent. Since fluconazole-resistant species have gained importance (14, 22, 23), and *Candida albicans* strains with decreased susceptibility to fluconazole have been described (14), it is important to use techniques which generate accurate and reproducible antifungal susceptibility test results.

Routine antifungal susceptibility testing is still not a recommended procedure (1), since most *Candida* species have a predictable susceptibility pattern and the reference test method, NCCLS broth macrodilution (9), is labor-intensive and therefore not readily applicable in routine laboratories with a high daily workload.

In the present study, the gold standard NCCLS broth macrodilution method (9) was compared with the Neo-Sensitabs method (Rosco, Taastrup, Denmark) (18) and the E-test (AB-Biodisk, Solna, Sweden) (12) for the determination of fluconazole susceptibility with different yeast species.

MATERIALS AND METHODS

Organisms. A total of 102 clinical yeast isolates were included: 11 strains from the H. Hartziekenhuis Roeselare, 18 strains from the Academisch Ziekenhuis Brussel, 24 strains from the Algemeen Ziekenhuis St. Jan Brugge, and 49 strains from the Ghent University Hospital, Ghent, Belgium. The following species were studied: *Candida glabrata* ($n = 30$), *C. albicans* ($n = 20$), *Candida tropicalis* ($n = 13$), *Candida parapsilosis* ($n = 10$), *Candida krusei* ($n = 8$), *Candida kefyr* ($n = 4$), *Candida guilliermondii* ($n = 4$), *Candida lusitaniae* ($n = 3$), *Candida norvegensis* ($n = 1$), *Candida lipolytica* ($n = 1$), *Candida humicola* ($n = 1$), *Candida pseudotropicalis* ($n = 1$), *Candida parakrusei* ($n = 1$), *Cryptococcus neoformans* ($n = 3$), and *Saccharomyces cerevisiae* ($n = 2$). Two quality control strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) and two reference strains (*C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 90018) were also included. All 106 isolates were identified to species level by internal transcribed spacer 2-PCR (20).

Inoculum preparation. Prior to testing, each isolate was grown on Sabouraud agar (Becton Dickinson, BBL, Heidelberg, Germany) for 24 h at 35°C. Suspensions were prepared in 0.85% saline to achieve a 0.5 McFarland standard by spectrophotometric measurement.

NCCLS broth macrodilution. Broth macrodilution testing was performed according to the NCCLS M27-A guidelines (10). Fluconazole (Pfizer, New York, N.Y.) was obtained as reagent-grade powder. Tubes containing twofold fluconazole dilutions, ranging from 0.25 to 256 mg/liter, in RPMI 1640 medium (Life Technologies, Gibco-BRL) and buffered with 0.164 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma, St. Louis, Mo.), were prepared in a single batch and stored frozen at −20°C until used. The macrobroth dilutions were incubated at 35°C and measured spectrophotometrically after 48 h (72 h for *C. neoformans*) to verify the presence or absence of growth. The turbidity was measured by diluting 0.2 ml of drug-free control growth with 0.8 ml of RPMI medium to produce an 80% inhibition standard (3).

Disk diffusion. Two milliliters of the 0.5 McFarland standard suspension was poured onto modified Shadomy agar (containing yeast nitrogen base, glucose, and asparagine) (Eδ-O Laboratories, Burnhouse, Scotland). A 15-μg fluconazole-containing tablet (Neo-Sensitabs; Rosco) was placed on the surface. The plates were incubated at 35°C, and zone diameters were read after 18 to 24 h (42 to 48 h for *C. neoformans*). The zones were measured to the diameter at which colonies of normal size occurred. The small and medium-sized colonies were considered nonresistant mutants, according to the manufacturer's guidelines.

E-test diffusion. E-tests were carried out on RPMI-agar plates, prepared by adding sterile liquid RPMI 1640 (4.6%) to Bacto agar (1.5%) (Difco Laboratories, Detroit, Mich.) plates, which were stored at 4°C for a maximum of 1 week. The 0.5 McFarland inoculum was swabbed in three directions on the entire RPMI-agar plate, and the E-test strip (AB Biodisk, Solna, Sweden) was applied. The plates were incubated at 35°C, and MICs were read after 24 h (48 h for *C. neoformans*). The MIC was read at the intersection (at the point of approximately 80% growth inhibition) of the zone edge and the E-test strip. Illustrations for the interpretation of the results, as provided by the manufacturer, were consulted.

Breakpoints. NCCLS breakpoints were used (9). Isolates were classified as susceptible if the MIC for the isolate was ≤8 mg/liter, susceptible-dose dependent if the MIC was ≥16 to ≤32 mg/liter, and resistant if the MIC was ≥64 mg/liter.

The Rosco criteria have recently changed, and only the more stringent diameters ($R \leq 16$ mm and $S \geq 30$ mm), formerly applicable only for systemic infections, are now advised for use.

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TABLE 1. Distribution of fluconazole MICs tested with the NCCLS macrobroth dilution and E-test methods

Species (no. of strains)	Test	No. of strains inhibited at fluconazole concn (mg/liter):											No. of noninterpretable results
		<0.25	0.25	0.5	1	2	4	8	16	32	64	128	≥256	
C. glabrata (30)	NCCLS						2	7	18	2			1	
	E-test						1	7	20				2	
C. albicans (21)	NCCLS		15	6										
	E-test	15	3	3										
C. tropicalis (13)	NCCLS			1	5	2							1	4
	E-test		1	4	2	2								4
C. parapsilosis (12)	NCCLS		1	6	3	1				1				
	E-test	2	2	3	4				1					
C. krusei (9)	NCCLS									7	2			
	E-test									3			6	
S. cerevisiae (2)	NCCLS					1	1							
	E-test							1	1					
Cryptococcus neoformans (3)	NCCLS		1			1	1							
	E-test				1			1	1					
Other *Candida*. spp. (16)	NCCLS		3	3	3	3	3			1				
	E-test	2	2	4	3	1	1	2	1					

Quality control. Quality control was performed by testing four American Type Culture Collection (ATCC) *Candida* strains: *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, used as quality control strains (11), were examined six times with all test methods; the two reference strains, *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 90018, were examined four or five times.

Interpretation of results. Statistical analysis was done by linear regression, correlating NCCLS MICs and inhibition zone diameters, respectively, with E-test MICs. Results were also analyzed in terms of clinical categorization. The percentage of very major errors was calculated as the number of susceptible strains (according to the evaluated method) that were indicated to be resistant by the reference method, divided by the number of resistant strains according to the reference method. Analogously, the percentage of major errors was calculated as the number of resistant strains (according to the evaluated method) that were indicated to be susceptible by the reference method, divided by the number of susceptible strains according to the reference method. The percentage of minor errors was calculated as the number of susceptible strains according to the evaluated method (or the number of resistant strains according to the evaluated method) that were indicated to be susceptible-dose dependent by the reference method, divided by the total number of strains, and vice versa (10).

RESULTS

The MICs for the quality control strains, as determined by the NCCLS broth macrodilution and the E-test methods, were within the ranges established by Pfaller et al. (11). Diameters observed for the fluconazole Neo-Sensitabs diffusion method were within the ranges indicated by the manufacturer.

The comparison of fluconazole MICs for 102 strains analyzed by the NCCLS method and the E-test resulted in a correlation coefficient of 0.943. Table 1 shows the distribution of fluconazole broth macrodilution and E-test MICs.

Four *C. tropicalis* strains did not give interpretable endpoints with either the NCCLS or the E-test because of substantial trailing. For 37% of the total number of strains tested, a double zone with growth of microcolonies throughout the ellipse zone was seen. This pattern was seen most often with *C. albicans* (18 of 21 strains [86%]) and *C. glabrata* (20 of 30 strains [67%]). Macrocolonies were seen with 5.6% (6 of 106) of the strains.

The E-test indicated lower mean MICs for *C. albicans*, *C. parapsilosis*, and *C. tropicalis* than did broth macrodilution, without changing the clinical interpretation according to the breakpoints used. However, one *C. tropicalis* strain with a MIC of >256 mg/liter by the reference method, appeared to be susceptible with the E-test (MIC = 0.5 mg/liter), resulting in a very major error. For the other *Candida* species tested, the E-test yielded lower mean MICs than the NCCLS method, but without a change in category. For *C. glabrata*, six of the nine susceptible strains were indicated to be susceptible-dose dependent, and one of the 20 susceptible-dose dependent strains was indicated to be resistant with the E-test. For *C. krusei*, four of the seven susceptible-dose dependent strains were indicated to be resistant with the E-test. One each of the two *S. cerevisiae* and three *C. neoformans* susceptible strains were indicated by the E-test method to be susceptible-dose dependent.

Thus, with the E-test, one very major error was observed (*C. tropicalis*, 25%), no major errors were found, and 23.5% minor errors were observed.

Figure 1 shows the correlation between the Neo-Sensitabs zone diameters and the NCCLS MICs. The correlation coefficient *r* was 0.873. No very major errors were seen, but major errors occurred with 14.1% of the strains tested. Using the breakpoints advised by the manufacturer, the Rosco tablet produced a minor error with the same *C. tropicalis* strain which produced a very major error with the E-test. In total, minor errors were observed with 29.4% of strains tested.

Lowering the disk diffusion breakpoints to ≤10 mm for resistance (*R*) and ≥21 mm for susceptibility (*S*) apparently resulted in a better correspondence between the disk diffusion and broth macrodilution methods (Fig. 1). There was a major error with only 1.4% of strains, and fewer minor errors (15.7%) were observed (Table 2). The *C. tropicalis* strain which was resistant by the NCCLS method but susceptible with the E-test was also indicated to be susceptible with the Neo-Sensitabs using the study criteria.

DISCUSSION

Since yeast infections are increasing and more resistant strains are observed (15, 16), a comparison of the currently

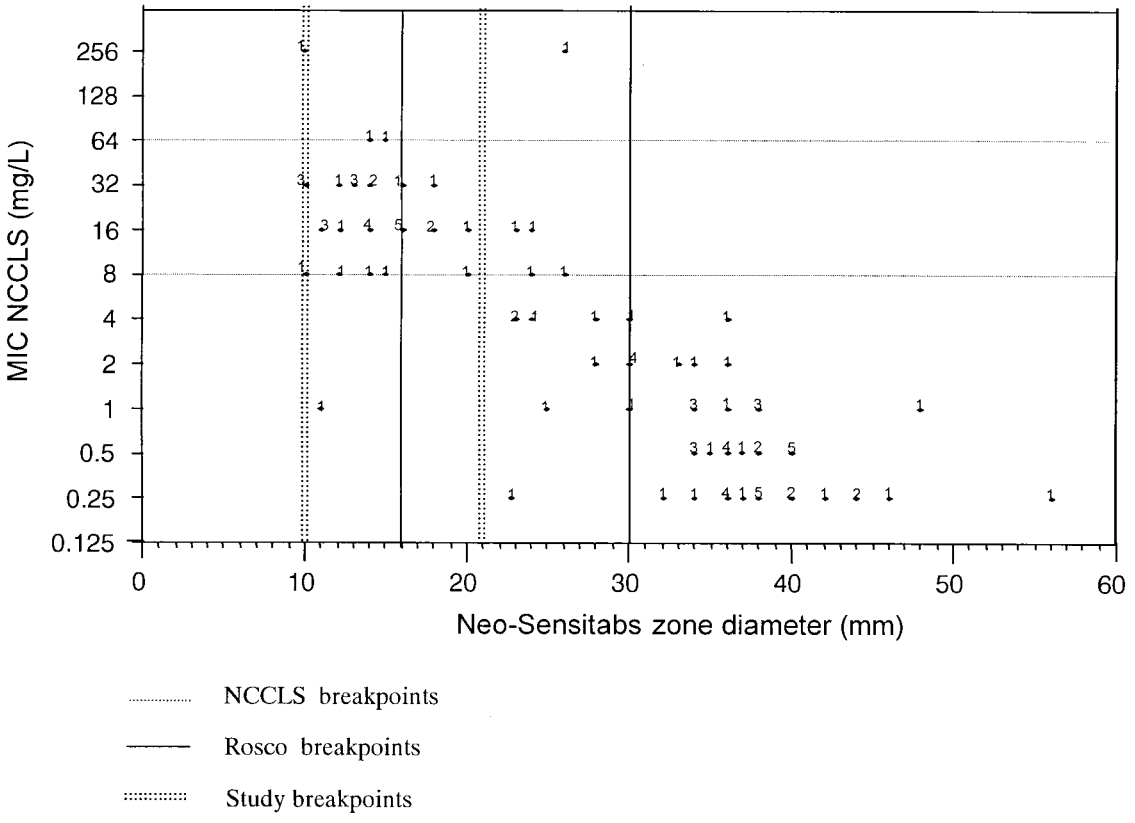

FIG. 1. Scatter diagram of NCCLS MICs for fluconazole and zone diameters obtained with 15-μg fluconazole disks.

available routine methods for fluconazole susceptibility testing with the gold standard method was undertaken.

The spectrophotometric reading performed in the NCCLS method provided an objective result. The subjective interpretation of minimal turbidity, as well as the trailing effect (seen particularly with *C. albicans*), was circumvented largely by producing an 80% inhibition standard (5, 13). However, interpretation of the precise meaning of "a prominent decrease in turbidity from the control" remains problematic (17). The MICs obtained corresponded with published data from the literature (2, 12, 18).

Several studies have shown that the E-test is an accurate method for MIC determination with numerous bacteria, including fastidious microorganisms and also *Candida* spp. (6, 7, 19). To minimize the problem of trailing endpoints due to partial inhibition by fluconazole, the E-test was performed on RPMI-agar with 2% glucose and the point of intersection was determined at 80% growth inhibition, as recommended previously (12). In addition, retesting the microcolonies from the inner growth zone showed that a greater degree of resistance for these isolates could be excluded. The distinction between these microcolonies and true macrocolonies is not always straightforward and requires experience.

The correlation of the E-test with NCCLS MICs was acceptable. However, to the best of our knowledge, no *r* values for fluconazole susceptibility testing of yeasts have been published. The MICs obtained were comparable to those published in other studies (6, 19). Only one *C. tropicalis* strain was resistant according to the NCCLS reference method but susceptible with the E-test (a very major error), but since only four resistant strains were available for testing, this caused a high very major error ratio (25%). To obtain a more realistic estimate of the very major error ratio, a larger number of fluconazole-resistant *C. tropicalis* strains should be tested. Minor errors were due to a shift towards higher susceptibilities with the E-test. Overall, lower mean MICs were observed with the E-test, and this is in agreement with previous work (21).

Four of the 13 *C. tropicalis* strains gave noninterpretable results with the NCCLS broth macrodilution method and the E-test because of substantial trailing and could not be included in the data set. These species-dependent difficulties have also been seen in other studies (3). To prevent variability in endpoint reading, plates were always scored by the same person. However, in a routine laboratory, trailing might constitute an important source of endpoint variability (4).

TABLE 2. Errors with the E-test and disk diffusion (two-diameter interpretation criteria) compared to the NCCLS broth macrodilution method[a]

Error type	No. (%) of strains		
	E-test	Rosco breakpoints ($R \leq 16$ mm, $S \geq 30$ mm)	Suggested breakpoints ($R \leq 10$ mm, $S \geq 21$ mm)
Very major error	1 (25)	0 (0)	1 (25)
Major error	0 (0)	10 (14.1)	1 (1.4)
Minor error	24 (23.5)	30 (29.4)	16 (15.7)

[a] According to the NCCLS broth macrodilution method, 69 strains were susceptible, 29 strains were susceptible-dose dependent, and 4 strains were resistant. Suggested breakpoints were determined, using data from Fig. 1.

The methodology will have to be adapted for testing of *S. cerevisiae* var. *boulardii* strains, since the two isolates in this study could not be grown on RPMI macrobroth or RPMI-agar for the E-test, although these isolates grew on Sabouraud agar.

For disk diffusion-based susceptibility testing carried out with Rosco tablets on modified Shadomy agar, the zone edge was taken where the colonies reached a normal size, and faint growth in the inhibition zone was disregarded.

Using the Rosco breakpoints ($R \leq 16$ mm and $S \geq 30$ mm), 14.1% major errors occurred. Using the lower breakpoints ($R \leq 10$ mm and $S \geq 21$ mm), deduced from the comparative study described in this paper (Fig. 1), fewer major errors (1.4%) were observed. Minor errors consisted of a shift towards lower susceptibilities with both sets of criteria. In general, lower mean diameter values were observed with the disk diffusion method.

The original breakpoints to be used, as proposed by the manufacturer of fluconazole tablets (Rosco), were based on different studies consisting of MIC determinations for isolates from patients treated with fluconazole daily (100 or >100 mg), on area under the curve/MIC determinations, and on clinical outcome. The present study used regression analysis to determine diameter breakpoints and did not take into account the pharmacokinetic aspects. The lower inhibition diameter breakpoints proposed here ($R \leq 10$ mm and $S \geq 21$ mm) are comparable to those established previously ($R \leq 12$ mm and $S \geq 20$ mm [8] and $R \leq 9$ mm and $S \geq 20$ mm [M. Vandevenne, I. Vandenbossche, G. Verschraegen, and L. Van Nimmen, Abstr. Natl. Symp. Belgische Vereniging Klinische Biologie-Société Belge de Biologie Clinique, abstr. 10, 2000]). Use of the diameter values proposed by Rosco caused too many major errors. By lowering those values, major error rates became more acceptable and fewer minor errors were seen. The Rosco tablet, used in combination with the diameter values proposed in this paper, appears to be an accurate alternative for the more expensive E-test.

In summary, the performance of susceptibility tests according to the NCCLS reference method on a large scale is technically difficult. The E-test is a very acceptable alternative. The disk diffusion method with the diameter values proposed by Rosco produced too many major errors. By lowering these values to $R \leq 10$ mm and $S \geq 21$ mm, acceptable major error rates and fewer minor errors were obtained, making the Rosco tablet a reasonable alternative for the more expensive E-test. More resistant strains, especially *C. tropicalis*, should be tested to put the percentage of very major errors into perspective.

As a final caution it should be emphasized that whatever technique is used, experience in determining MICs and the interpretation of inhibition zone diameters, together with careful attention to procedure details, is critically important when performing fluconazole susceptibility testing for yeasts.

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