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Comparison of Two Alternative Microdilution Procedures with the National Committee for Clinical Laboratory Standards Reference Macrodilution Method M27-P for In Vitro Testing of Fluconazole-Resistant and -Susceptible Isolates of *Candida albicans*

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The National Committee for Clinical Laboratory Standards has proposed a reference broth macrodilution method for in vitro antifungal susceptibility testing of yeasts (the M27-P method). This method is cumbersome and time-consuming and includes MIC endpoint determination by the visual and subjective inspection of growth inhibition after 48 h of incubation. Two alternative microdilution procedures for MIC endpoint determination, a spectrophotometric MIC endpoint test that evaluates 80% growth inhibition by the drug and a colorimetric method with an oxidation-reduction indicator (Alamar Blue), were compared with the M27-P method for fluconazole susceptibility testing of 45 susceptible and resistant isolates of *Candida albicans*. The spectrophotometric method was performed with RPMI 1640 medium with 2% glucose, and the other two tests were performed with plain RPMI 1640 medium. All tests were incubated at 35°C. Excellent agreement was demonstrated between the M27-P method and both 24-h microdilution tests (97.7%) as well as between the two microdilution tests (95.5%). Also, there was agreement in the detection in vivo of fluconazole resistance by the three methods. These preliminary data indicate that both microdilution methods may serve as less subjective alternatives to the M27-P method for the determination of fluconazole MIC endpoints.

Since the late 1940s the frequent use of chemotherapy including antibiotics and cortisone have been responsible for the higher incidence of severe fungal infections (12, 24, 26). In more recent years, alterations in the host defense mechanisms as a consequence of human immunodeficiency virus infection, more intensive and aggressive cytotoxic chemotherapy, and trauma have predisposed AIDS, oncology, and transplantation patients to invasive infectious diseases caused by fungi. One trend has been an increase in the incidence of nosocomial yeast infections (2). Although *Candida albicans* is considered the most prevalent species recovered from humans, during the last 20 years a marked shift in the spectrum of *Candida* infections among patients with different types of cancer has been noted. In a greater proportion (35 to 46%) of these patients with systemic candidiasis, other *Candida* species have replaced *C. albicans* as the etiologic agents of infection, and this trend also has been reported among nononcology patients (20, 25). These non-*C. albicans* species are usually less susceptible to antifungal therapy. The increased incidence and severity of these mycoses prompted the development and more frequent use of antifungal agents, which also induced the replacement of susceptible fungi by less susceptible organisms.

As a result of these combined factors, the performance of antifungal susceptibility testing has become very important in the clinical laboratory. Although methodologies for in vitro susceptibility testing have been available since the early years of antifungal drug development (23), such procedures lacked standardization, and reproducibility among the laboratories was low until the early 1990s (5, 9, 10, 19). After 10 years of collaborative investigations (5, 9, 19), the Subcommittee on Antifungal Susceptibility Tests of the National Committee for Clinical Laboratory Standards (NCCLS) proposed in 1992 a broth macrodilution method for the testing of yeasts (the NCCLS M27-P method) (13). This method is cumbersome and time-consuming, and the MIC endpoint determination, its most critical step, relies on the subjective and visual evaluation of growth inhibition. During the last 3 years, alternative methods have been developed and evaluated (1, 3–7, 16–18, 21). Most of these procedures follow a microdilution format which is to be included in the future NCCLS M27-T document (14). Earlier studies have compared MICs that represented 50% drug inhibition against the 80% inhibition evaluated by the M27-P reference method (1, 18) and included isolates for which well-documented in vivo response data were unavailable. The purpose of the present study was to compare two broth microdilution antifungal susceptibility tests, a spectrophotometric MIC endpoint test that evaluates 80% growth inhibition (MIC-80% endpoint test) and a colorimetric MIC procedure with the indicator Alamar Blue, with the NCCLS M27-P method in detecting potential fluconazole-susceptible and -resistant isolates of *C. albicans*. Twenty-six of the 45 clinical isolates tested were recovered from patients for which well-documented fluconazole therapy data were available (16 patients failed therapy and 10 patients responded to therapy).

MATERIALS AND METHODS

Test organisms. The 50 test organisms included 45 clinical isolates of *C. albicans*. These isolates were recovered from 45 different AIDS patients with oral

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candidiasis from 12 hospitals in Spain. Twenty-six of these patients had well-documented clinical cases of oropharyngeal and/or esophageal candidiasis who either failed (16 patients) or responded (10 patients) to fluconazole therapy. Some of these patients have been described elsewhere (22). In vitro susceptible (*Candida parapsilosis* ATCC 90018, *C. albicans* ATCC 90028, and *C. albicans* ATCC 64548) and resistant (*C. albicans* ATCC 64550 and *Candida krusei* ATCC 6258) test organisms also were evaluated and served as quality control (QC) organisms. For three of these strains (ATCC 90018, ATCC 90028, and ATCC 6258) reference MIC ranges of amphotericin B, flucytosine, and fluconazole are well defined (15). The two former strains have been recommended by NCCLS as reference isolates and the latter has been recommended as one of the two QC strains for antifungal susceptibility testing of yeasts. Each isolate was maintained in sterile water at ambient temperature in each laboratory until testing was performed.

Antifungal agent. The MICs of fluconazole (Roerig Pfizer, New York, N.Y.) were determined by the broth macrodilution procedure (the M27-P method) and two alternative broth microdilution procedures, a colorimetric test with the indicator Alamar Blue and a spectrophotometric procedure. Fluconazole was obtained from the manufacturer as a reagent-grade powder and was dissolved in sterile distilled water as 100× stocks that were frozen at −70°C until they were used.

NCCLS macrodilution reference method (M27-P). The broth macrodilution test was performed by following the guidelines of the proposed reference method for yeasts (NCCLS document M27-P) (13). The medium used to prepare the 10× drug dilutions and inoculum suspensions was RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Whittaker Bioproducts, Inc., Walkersville, Md.). The yeast suspensions used as inocula were prepared by the spectrophotometric procedure and ranged from 0.6×10^3 to 2.1×10^3 CFU/ml. Yeast inoculum (0.9 ml) was added to each test tube, which contained 0.1 ml of a 10× fluconazole concentration. The final concentrations of the antifungal agent ranged from 0.12 to 64 μg/ml. The growth control tube(s) contained a 0.9-ml volume(s) of an inoculum suspension and a 0.1-ml volume(s) of drug-free medium. The American Type Culture Collection (ATCC) strains were tested in the same manner as the clinical isolates. In addition, 1 ml of uninoculated, drug-free medium was included as a sterility control.

Broth macrodilution MICs were determined after 48 h of incubation at 35°C by visually comparing the turbidity of each MIC tube with an 80% inhibition standard (1:5 dilution of the corresponding growth control) (8, 13). Macrodilution MIC-80% endpoints were the lowest drug concentration with a turbidity less than or equal to that of the standard.

Microdilution spectrophotometric MIC-80% method with RPMI 1640 medium–2% glucose. The microdilution spectrophotometric MICs were determined according to the guidelines of the M27-P document and the following modifications: RPMI 1640 medium–2% glucose (21), inocula of 10^4 CFU/ml (4), incubation for 24 h, and spectrophotometric reading of an MIC-80% endpoint (22). The preparation of the RPMI 1640 medium–2% glucose has been described previously (21). Briefly, the buffered (MOPS) RPMI 1640 broth was prepared in 900 ml of distilled water and was filter sterilized. Following sterilization, 100 ml of an autoclaved solution of glucose (180 g/liter) was added. This step brought the concentration of glucose to 2%. Yeast inocula (approximately 10^6 CFU/ml) were prepared by the spectrophotometric procedure, and the microdilution trays were inoculated with an automatic pipettor programmed to deliver 10 μl to each well, which contained a 100-μl volume of the drug at various concentrations (range, 0.12 to 64 μg/ml). This step brought the inoculum to the final test density of approximately 10^4 CFU/ml. The growth control well(s) contained a 100-μl volume(s) of RPMI 1640 broth and a 10-μl volume(s) of inoculum; the MICs for the ATCC strains were determined as described above for the clinical isolates.

Following incubation at 35°C for 24 h, the microdilution trays were shaken for 5 min and spectrophotometric readings of each well were performed at 405 nm with a Mios Merck automatic plate reader (Merck Igoda, S.A., Madrid, Spain). The spectrophotometric MIC endpoint was calculated from the turbidimetric data as the lowest drug concentration giving rise to an inhibition of growth equal to or greater than 80% of that of the drug-free control (MIC-80%). This concept is similar to the visual endpoint criterion recommended in the reference method.

Microdilution colorimetric method with Alamar Blue. The microdilution trays containing serial dilutions of dried fluconazole (0.12 to 64 μg/ml) and the colorimetric oxidation-reduction indicator Alamar Blue were prepared by Alamar Biosciences (Sacramento, Calif.). Each well of the panel was rehydrated by inoculating it with a 100-μl volume(s) of the inoculum suspension in RPMI 1640 medium buffered (MOPS) to pH 7.0 (Whittaker). The final inoculum densities ranged from 0.9×10^3 to 2.4×10^3 CFU/ml. The growth control well(s) contained a 100-μl volume(s) of inoculum; the MICs of the QC ATCC strains were determined in the same manner.

Broth microdilution colorimetric MICs were read visually after 24 and 48 h of incubation at 35°C by observing a change in color from blue (negative) to purple or red (positive). The MIC was the lowest drug concentration showing no color change (first blue well) or a slight color change from blue to purple.

TABLE 1. Fluconazole MICs determined by the NCCLS broth macrodilution reference method (M27-P) and two alternative microdilution tests for five control ATCC strains[a]

Organism and ATCC strain[b]	MIC (μg/ml) by the following method:		
	NCCLS	Spectrophotometric	Colorimetric
C. parapsilosis ATCC 90018	1.0	0.5	4.0
C. albicans ATCC 90028	0.5	0.25	0.25
C. albicans ATCC 64548	0.5	0.25	1.0
C. albicans ATCC 64550	16	32	16
C. krusei ATCC 6258	32	32	32

[a] The NCCLS broth macrodilution M27-P reference method was used. The alternative methods were the microdilution spectrophotometric method with RPMI 1640 medium–2% glucose (MIC-80%) and microdilution colorimetric method with Alamar Blue.

[b] The strains were of group 1, as defined in the text. MIC references ranges have been established for the ATCC 90018, ATCC 90028, and ATCC 6258 strains (15).

RESULTS

The 50 test organisms (45 clinical isolates and 5 ATCC strains) were classified into four groups according to the fluconazole MIC endpoints and in vivo responses to fluconazole therapy (Tables 1 and 2). Group 1 contained the five ATCC isolates (Table 1); group 2 contained the isolates of *C. albicans* for which fluconazole MICs were ≤1.0 μg/ml (in vitro and in vivo susceptible strains which included 10 isolates from patients who responded to fluconazole therapy); group 3 contained *C. albicans* isolates for which fluconazole MICs were ≥2 μg/ml; and group 4 contained isolates of *C. albicans* from patients who were fluconazole treatment failures (in vivo resistant strains) (Table 2). For all comparisons of MIC endpoints, MICs were considered in agreement when the differences between the values were within 2 doubling drug dilutions (±1 dilution). Table 1 summarizes the fluconazole MICs for the five ATCC isolates determined by the three in vitro antifungal susceptibility testing procedures. Fluconazole MICs for the NCCLS reference isolates *C. parapsilosis* ATCC 90018, *C. albicans* ATCC 90028, and the QC isolate *C. krusei* ATCC 6258 were within the established reference fluconazole ranges of 0.25 to 1.0 μg/ml and 16 to 64 μg/ml by the macrodilution and microdilution spectrophotometric procedures, respectively. The colorimetric fluconazole MIC (4 μg/ml) for *C. parapsilosis* ATCC 90018 was outside of the reference range. However, both alternative microdilution tests provided MICs either identical or similar (within 2 dilutions) to the one obtained by the reference method (M27-P) for all five ATCC isolates.

Table 2 summarizes the 24-h fluconazole MICs obtained by both microdilution tests and the 48-h MIC endpoints obtained by the macrodilution reference method for the three groups of *C. albicans* isolates. Comparison of MICs for the 45 clinical isolates demonstrated an excellent overall agreement (98%) between the MICs obtained by the reference method and those obtained by both microdilution tests at 24 h. Values were lower when the 48-h colorimetric MICs were compared with the M27-P endpoints (data not shown in Table 2). Comparison between the two microdilution tests provided a 96% agreement. For clinical isolates of *C. albicans* for which fluconazole MICs were less than 1.0 μg/ml (group 1), the agreement between the macrodilution reference method and the alternative microdilution procedures as well as between the two microdilution tests was 100%. Discrepancies between the tests were found among the fluconazole MICs for the *C. albicans* isolates

TABLE 2. Fluconazole MICs determined by the NCCLS broth macrodilution reference method and two alternative microdilution tests for 45 susceptible and resistant clinical *C. albicans* isolates[a]

Group, organism, and isolate code no.	MICs (μg/ml) by the following method:		
	NCCLS	Spectrophotometric	Colorimetric
Group 2. *C. albicans* (MICs, ≤1.0 μg/ml)[b]			
1. CL511	0.12	0.12	0.25
2. CL381	0.12	0.12	0.25
3. CL205	0.12	0.25	0.25
4. CL427	0.12	0.25	0.25
5. CL687	0.12	0.25	0.25
6. CL386	0.25	0.12	0.5
7. CL256	0.25	0.12	0.25
8. CL456	0.25	0.12	0.25
9. CL705	0.25	0.25	0.5
10. CL505	0.5	0.25	0.5
11. CL373	0.5	0.5	0.5
12. CL466	1.0	0.5	0.25
Group 3. *C. albicans* (MICs, ≥2.0 μg/ml)			
1. CL436	2	4	4
2. CL236	4	1	2
3. CL626	4	4	4
4. CL922	4	8	4
5. CL389	4	>64	8
6. CL949	8	8	8
7. CL1186	8	8	16
8. CL1113	8	16	16
9. CL735	8	16	16
10. CL1367	8	16	16
11. CL1383	16	16	16
12. CL1022	16	32	16
13. CL468	32	32	32
14. CL152	64	>64	>64
15. CL1139	64	>64	64
16. CL958	>64	>64	>64
17. CL341	>64	>64	>64
Group 4. *C. albicans* from fluconazole treatment failures			
1. CL1026	4	8	4
2. CL1023	8	8	8
3. CL342	8	16	8
4. CL668	8	16	16
5. CL286	8	32	16
6. CL589	8	32	16
7. CL1147	16	16	2
8. CL351	32	8	8
9. CL191	32	32	16
10. CL282	32	32	16
11. CL275	32	64	64
12. CL184	32	>64	32
13. CL585	32	>64	64
14. CL1225	32	>64	64
15. CL99	64	>64	32
16. CL257	64	>64	32

[a] The NCCLS broth macrodilution M27-P reference method was used. The alternative methods were the microdilution spectrophotometric method with RPMI 1640 medium–2% glucose (MIC-80%) and the microdilution colorimetric method with Alamar Blue at 24 h.

[b] Ten of the 12 isolates in group 2 were recovered from patients who responded to fluconazole therapy.

from groups 3 and 4. That is, for isolate 5 of group 3 the MIC was >64 μg/ml by the spectrophotometric method and the MICs were 4 to 8 μg/ml by the other two tests. On the other hand, for isolate 7 from group 4 the colorimetric MIC was 2 μg/ml but the MICs were 16 μg/ml by both the reference and the spectrophotometric procedures. With the exception of the colorimetric MIC given above (for isolate 7), the fluconazole MIC ranges for the 16 isolates recovered from the patients nonresponsive to fluconazole therapy were 4 to >64 μg/ml by the three methods.

DISCUSSION

Ideally, antimicrobial susceptibility testing should be easy to perform and cost-effective and should provide MIC endpoints which are reproducible and readily determined after a short incubation period. In addition, these MIC results should serve as predictors of drug efficacy or in vivo response. In the field of antifungal susceptibility testing, it was not until the early 1990s that good to excellent interlaboratory agreement of MIC data was achieved (5, 9, 19). This was accomplished by the proposal in 1992 of a broth macrodilution reference method (the NCCLS M27-P method) for the antifungal susceptibility testing of yeasts (13). Nevertheless, this method has several drawbacks.

In general, a broth macrodilution test is not a convenient and practical procedure to be used in the routine clinical laboratory. Because of this, we compared two alternative procedures, a spectrophotometric test and a colorimetric test, for the in vitro antifungal susceptibility testing of *C. albicans* isolates. Both alternative procedures followed a microdilution format, which is a more efficient and convenient tool in the clinical laboratory. Furthermore, the microdilution approach has compared favorably in earlier studies with the M27-P method with plain RPMI 1640 medium (NCCLS microdilution test) (5) or RPMI 1640 medium supplemented with the oxidation-reduction colorimetric indicator Alamar Blue (16, 17). In our study, colorimetric microdilution MICs matched most of the values obtained by the NCCLS macrodilution method when testing the 5 ATCC strains (Table 1) and the 45 clinical isolates of *C. albicans* (Table 2). The only exceptions were the colorimetric MICs for both of the NCCLS reference strains of *C. parapsilosis* (Table 1) and the *C. albicans* strain coded as strain 7 (group 4, fluconazole therapy failures). The colorimetric MIC for the latter isolate was 2 μg/ml, whereas the macrodilution and spectrophotometric MICs were 16 μg/ml (Table 2). The MIC of 2 μg/ml could be related either to the subjectivity of the procedures or, more likely, to the limited growth of this strain after 24 h of incubation, because its MIC at 48 h was 16 μg/ml (data not shown in Table 2).

The colorimetric test was performed with RPMI 1640 broth, the NCCLS testing medium, which has been noted to provide suboptimal growth for certain species of yeasts (5, 9, 21). This effect is probably due in part to the low glucose concentration (0.2%) in this broth. Because of this, an incubation of 48 h has been established by the NCCLS as the optimal incubation time for the determination of MICs for *Candida* spp. and *Torulopsis glabrata* and an incubation of 72 h has been established for *Cryptococcus neoformans*. However, previous studies (5, 16, 17) and data from the present study appear to indicate that an incubation of 48 h may not be necessary for isolates of *C. albicans* by the microdilution test. With the exception of isolate 7 (group 4), 24-h colorimetric MICs matched the macrodilution MICs better than the 48-h values did. A shorter incubation period is one of the advantages of the microdilution methodology.

Another drawback of both NCCLS macro- and microdilu-

tion tests is that the critical MIC endpoint determination involves the visual and subjective examination of growth inhibition. Although this step creates very little problem when testing amphotericin B, the evaluation of the partial inhibition observed when testing flucytosine and the azoles could become controversial. The determination of the macrodilution MICs of the azoles has been facilitated by the comparison of the growth inhibition in each MIC tube to an 80% inhibition standard (8, 13). The microdilution approach, even with the aid of the colorimetric indicator, still relies on the subjective interpretation of prominent growth inhibition or slight color change to define the MIC. On the other hand, the turbidimetric procedure, the second alternative evaluated in our study, is a more objective and efficient approach. The turbidimetric methodology has been applied for macrodilution antifungal testing since 1976 (11). Ensuing studies have demonstrated that turbidimetric microdilution MICs also compared favorably with the M27-P reference MICs (1, 18). These early comparisons reflected levels of growth inhibition of between 50 and 70%. The spectrophotometric method (21, 22) evaluated in the present study represents the same percentage (80%) of inhibition as the one recommended by NCCLS for flucytosine and the azoles. Furthermore, the increased concentration of the glucose content of the RPMI 1640 medium (to 2%) and the higher inoculum density (approximately 10^4 CFU/ml) optimized the growth of the isolates tested. The MICs were then evaluated at 24 h. Turbidimetric MICs determined by this methodology matched the NCCLS reference method endpoint MICs with only one exception (isolate 5 of group 2). In this case, the spectrophotometric test provided a higher (>64 μg/ml) MIC than the other two tests (4 to 8 μg/ml) (Table 2). The higher MIC could be related to an increased growth in the RPMI 1640 medium because of its higher content of glucose.

An earlier investigation demonstrated that the results of in vitro susceptibility testing obtained by this spectrophotometric procedure correlated with the clinical response to fluconazole and ketoconazole therapy for oropharyngeal and/or esophageal candidiasis associated with AIDS (22). Since we tested in our study 26 isolates from patients who either failed (16 isolates for which MICs were ≥4 μg/ml) or responded (10 isolates for which MICs were ≤1.0 μg/ml) to fluconazole therapy (100- to 400-mg oral dose), the utilities of these three procedures in detecting both in vivo and in vitro fluconazole-resistant and -susceptible strains were demonstrated for this patient population and setting.

In conclusion, great progress in the methodologies available for antifungal susceptibility testing of yeasts has been achieved. Both alternative microdilution procedures evaluated in the present study provided faster (24 h) and more easily determined MIC endpoints than the values obtained by the NCCLS M27-P reference method; those former values matched the latter test results. However, there is a trade-off for each of these alternatives. The colorimetric test does not require special laboratory equipment for the interpretation of MICs, but it is a more subjective test than the spectrophotometric procedure. On the other hand, the more objective turbidimetric evaluation of in vitro antifungal susceptibility requires a spectrophotometer, a piece of equipment that is not available in many clinical laboratories. The utility of antifungal susceptibility testing will be determined when the relationship between in vitro results and clinical response to therapy has been well established. Therefore, additional studies are essential in order to develop interpretive standards of in vitro data.

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