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Use of a Colorimetric System for Yeast Susceptibility Testing

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We examined the reliability and accuracy of a colorimetric assay using Alamar Blue reagent in the performance of susceptibility tests for *Candida albicans*. We compared the broth macrodilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) with a macrodilution method modified with the Alamar reagent and a microdilution method modified with the Alamar reagent. The MICs of fluconazole and itraconazole for 97 isolates of *C. albicans* and 3 control isolates were tested. For fluconazole, the Alamar-modified broth macrodilution method yielded 94% (91 of 97) concordance within 2 dilutions compared with the NCCLS method, while the microdilution method yielded 95% (92 of 97) concordance. With Alamar-modified methods for itraconazole, broth macrodilution yielded 97% (94 of 97) concordance within 2 dilutions. MICs obtained by the microdilution method, although tightly nested, were shifted to a higher value when compared with those obtained by the NCCLS method; there was only 77% (75 of 97) concordance within 2 dilutions but 97% concordance (94 of 97) within 3 dilutions. Tests by all methods with quality control strains showed excellent reproducibilities. For fluconazole, the methods modified with the Alamar reagent yielded clear endpoints and excellent correlation for the broth macrodilution and microdilution methods. For itraconazole, the methods modified with the Alamar reagent yielded clear endpoints and were reproducible, but higher MICs were obtained by the microdilution methods compared with those obtained by the NCCLS methods.

Recent studies have shown the utility of using oral and parenteral triazole antifungal agents for the prophylaxis and treatment of systemic fungal infections (4, 5, 7, 8, 15). Despite the increased use of the triazoles, in vitro susceptibility testing is infrequently performed and such tests are performed at only a few centers. Factors contributing to the infrequent use of in vitro testing include subjective determination of endpoints because of trailing, the cumbersome and time-intensive performance of the broth macrodilution assay recommended by the National Committee for Clinical Laboratory Standards (NCCLS), and the lack of established breakpoints for the azoles (6, 9, 14). Although many uncertainties exist regarding the clinical usefulness of antifungal susceptibility testing, the emergence of resistance to triazoles has prompted increased interest in antifungal susceptibility testing (2, 12, 16). Development of a simple, reproducible method which is less cumbersome to perform than the NCCLS broth macrodilution technique may aid in the selection of antifungal agents for the treatment of fungal infections.

With the assay recommended by NCCLS, MICs are determined by visually comparing the turbidity of each tube with that of a tube containing a 1:5 dilution of the growth control. Thus, the MIC is the lowest concentration of drug that inhibits growth by 80%. However, MIC endpoints are often problematic; trailing endpoints and particulate growth formation render evaluation of turbidity difficult. A method which incorporates a colorimetric indicator could have utility if it produced clearly demarcated endpoints.

Alamar Blue (Alamar Biosciences, Sacramento, Calif.), an oxidation-reduction colorimetric indicator, changes hue from a deep blue to a bright pink in the presence of metabolically active, growing organisms. The use of Alamar Blue has been evaluated most extensively for susceptibility testing of gram-negative bacilli, with a few recent studies assessing its use for susceptibility testing of yeasts (1, 10, 11, 13). We examined the feasibility and reliability of using an Alamar Blue-based method for yeast susceptibility testing by comparing the NCCLS standard broth macrodilution method with the same protocol modified by the addition of the colorimetric indicator and also with a microdilution assay with the indicator.

MATERIALS AND METHODS

Antifungal drugs. Fluconazole powder was obtained from Pfizer-Roerig, Inc. The stock solution was made by dissolving the powder in sterile distilled water and was then divided into 1-ml aliquots at a concentration of 5,120 μg/ml and frozen at −70°C. The stock solution was thawed, and fresh dilutions were made in sterile distilled water on each day that testing was performed.

Itraconazole powder was obtained from Janssen Research Foundation. The stock solution was made by dissolving the powder in dimethyl sulfoxide (DMSO) and was then divided into 1-ml aliquots at a concentration of 1,280 μg/ml and frozen at −70°C. The stock solution was thawed, and fresh dilutions were made by diluting the stock solution in warmed (35°C), sterile distilled water and vigorously mixing until the solution was completely dissolved. Fresh dilutions were made on each day that testing was performed.

Fungal isolates. Ninety-seven oropharyngeal *Candida albicans* isolates obtained from patients with human immunodeficiency virus infection over a time period of 2 years were selected for testing. Isolates were stored in Sabouraud dextrose broth and glycerol at −70°C until they were used. These isolates were then thawed and plated onto Sabouraud dextrose agar prior to testing. Three control strains were tested on each day that MICs were determined: two *C. albicans* strains (ATCC 90029 and ATCC 90028) and one *Candida krusei* strain (isolated from a patient).

MIC testing methods. On each day that susceptibility testing was performed, 21 isolates and 3 control strains were studied by three different methods: the NCCLS broth macrodilution assay, the NCCLS broth macrodilution assay with Alamar Blue added, and the broth microdilution assay with Alamar Blue added. The NCCLS broth macrodilution method was performed as recommended in the M27-P document (9). Succinctly, testing was performed with RPMI 1640 medium (Sigma, St. Louis, Mo.) with morpholinepropanesulfonic acid (MOPS) buffer (Sigma) at a pH of 7.0. Five monomorphic colonies of ≥1 mm diameter were taken from a culture grown at 35°C for 48 h and were suspended in 0.85% saline. Cell density was then adjusted to the turbidity of a 0.5 McFarland standard by the addition of saline. The final inoculum of 0.5×10^3 to 2.5×10^3 cells per ml was made in RPMI 1640–MOPS medium, and the suspension was added in individual 0.9-ml aliquots to glass tubes (13 by 100 mm). To each tube except the growth control tube 0.1 ml of drug was added to achieve final concentrations

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TABLE 1. Concordance of Alamar Blue-based MIC determinations with NCCLS standard broth macrodilution method

Alamar Blue method	Concordance with NCCLS method[a]		No. of strains for which Alamar Blue-based method results differed from those of NCCLS method	
	±2 dilutions	±3 dilutions	>2 dilutions	>3 dilutions
Fluconazole				
Macrodilution	91/97 (94)	95/97 (98)	6	2
Microdilution	92/97 (95)	95/97 (98)	5	2
Itraconazole				
Macrodilution	94/97 (97)	96/97 (99)	3	1
Microdilution	75/97 (77)	94/97 (97)	22	3

[a] Number of strains for which results by the Alamar Blue-based method agreed with those obtained by the NCCLS method/number of strains tested by both methods (percent).

of fluconazole of 0.06 to 64 μg/ml and of itraconazole of 0.006 to 6.4 μg/ml. The tubes were incubated in ambient air at 35°C for 46 to 50 h. The MIC was determined to be the minimum drug concentration at which visual turbidity was equal to or less than that of a dilution showing 80% growth suppression.

The Alamar Blue-modified NCCLS method was identical to the method described above in terms of inoculum, medium preparation, drug dilution, and incubation, with the exception that Alamar Blue (1 μl of 100× reagent) was added to each 1 ml of RPMI 1640–MOPS medium prior to inoculation with the organism. The MIC was determined as the lowest drug concentration that maintained a blue or a blue-pink hue. Tubes with growth were pink or red in color.

For the microdilution method, inoculum, medium preparation, and the addition of Alamar Blue to the RPMI 1640–MOPS solution were identical to those for of the Alamar Blue-modified NCCLS method. However, 96-well, clear U-bottom microtiter plates were used. Into each well, 180 μl of the RPMI 1640–MOPS–Alamar Blue solution containing 0.5×10^3 to 2.5×10^3 yeast cells per ml was pipetted, and 20 μl of the appropriate concentration of drug was added. The plates were agitated for 30 s and were then incubated for 46 to 50 h at 35°C before the results were read visually. The MIC was determined as the lowest drug concentration that maintained a blue or a blue-pink hue.

RESULTS

The MICs of fluconazole obtained by the Alamar Blue-modified NCCLS broth macrodilution method correlated well with those obtained by the standard NCCLS method. Overall, for 91 of 97 isolates (94%) the MICs were within 2 dilutions of those obtained by the NCCLS method (Table 1). The Alamar Blue-modified microdilution method showed similar agreement (95% within 2 dilutions) with the NCCLS standard method. When the Alamar Blue-modified macrodilution and microdilution methods were compared with each other, MICs for 95 of 97 isolates (98%) were within 2 dilutions and MICs for 93 of 97 isolates (96%) were within 1 dilution (Fig. 1).

For itraconazole, the Alamar Blue-modified and standard NCCLS broth macrodilution methods yielded MICs within 2 dilutions for 94 of 97 isolates (97%) (Table 1). However, by the Alamar microdilution method, the MICs for only 75 of 97 isolates (77%) were within 2 dilutions of the MICs determined by the NCCLS method. This discrepancy was almost entirely due to the MICs for 19 isolates, which differed by 3 dilutions. The MICs obtained by the microdilution method were greater by 1 to 2 dilutions compared with those obtained by the NCCLS method. The Alamar Blue-modified macrodilution and microdilution methods revealed a close correlation, with MICs for 97% of isolates (94 of 97) concurring within 2 dilutions (Fig. 1).

The two Alamar Blue-based methods were highly reproducible when MICs for control strains were compared from day to day. The MICs of both drugs for each of two *C. albicans* control strains were assayed by the two different methods on 6 different days. The fluconazole MICs always fell between 0.12 and 0.5 μg/ml, which is the acceptable MIC range for these two strains according to the M27-P document (9). One exception occurred when the MIC for one strain was 1.0 μg/ml on one determination. Itraconazole MICs ranged between 0.05 and 0.2 μg/ml by both methods. On 12 separate occasions, the

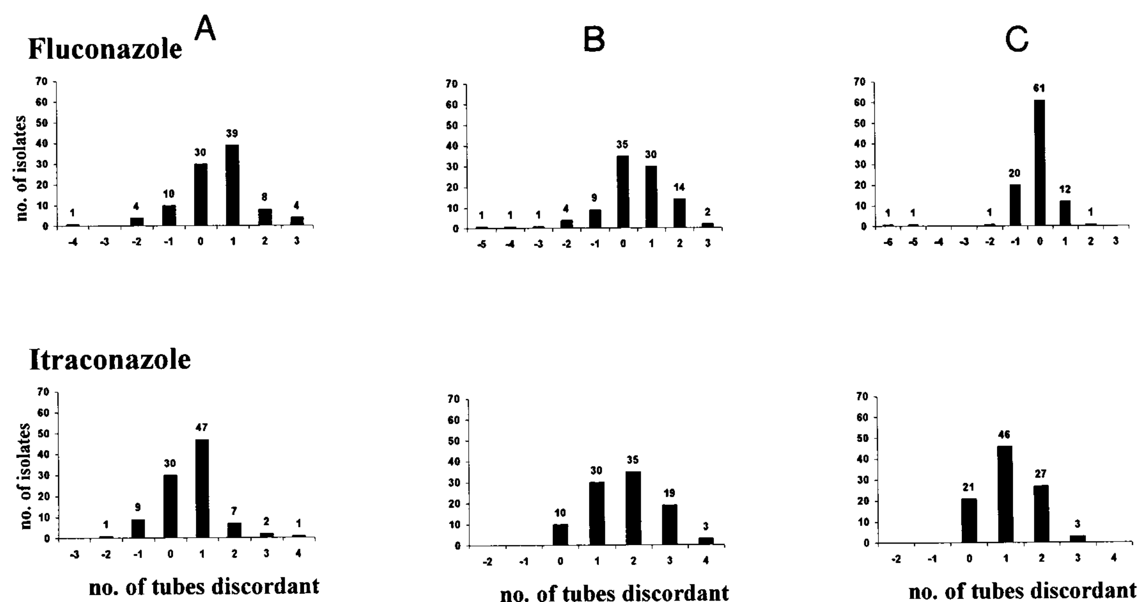

FIG. 1. Concordance of MICs among Alamar Blue-modified broth macrodilution and microdilution methods and NCCLS proposed standard M27-P (9). (A) Alamar Blue-modified macrodilution versus NCCLS method. (B) Alamar Blue-modified microdilution versus NCCLS method. (C) Alamar Blue-modified microdilution versus Alamar Blue-modified macrodilution. One datum point was deleted from the representation of the Alamar Blue-modified macrodilution method versus the NCCLS method for fluconazole because of contamination.

fluconazole MIC for the *C. krusei* strain was ≥64 μg/ml, and the itraconazole MIC was 0.4 to 0.8 μg/ml. Growth of organisms in the highest concentrations of itraconazole was noted by the NCCLS broth macrodilution assay, but no color change was noted in the Alamar Blue-modified methods at similar concentrations. This phenomenon was not observed in the fluconazole assays.

DISCUSSION

Alamar Blue is a unique colorimetric indicator that has been used to monitor tissue culture viability and has been evaluated for use in susceptibility testing of gram-negative bacteria. It has tetrazolium-like properties in that its light absorbance and emission spectra change when they are reduced by a biological cytochrome system. Chemical reduction is readily achieved in the presence of actively metabolizing prokaryotic or eukaryotic cells. These characteristics make it an ideal candidate for use in antifungal susceptibility testing, which has remained problematic despite great efforts to standardize a reliable, reproducible testing method.

Pfaller et al. (11) found a level of agreement of the MICs between the NCCLS broth macrodilution method and an Alamar Blue-modified microdilution method within 2 dilutions for 91 to 94% of *C. albicans* isolates, depending on the length of incubation. Our results confirm these findings for our *C. albicans* isolates, for which the fluconazole MICs covered a broad range (0.02 to ≥64 μg/ml). Alamar Blue-modified broth microdilution assays for fluconazole appear to be as reliable and reproducible as NCCLS standard methods (95% correlation with NCCLS determinations within 2 dilutions).

A recent multicenter study confirmed the independent reproducibility of an Alamar Blue-modified microdilution assay for measuring the fluconazole and amphotericin B susceptibilities of several *Candida* species (13). Another recent study showed the applicability of this method for measuring the MICs of fluconazole, amphotericin B, and flucytosine for many candidal and cryptococcal species (10).

Although the NCCLS M27-P standard (9) does not address the conditions for determining itraconazole MICs, we found that the adaptation of the methods suggested for fluconazole were highly reproducible with itraconazole. In general, the MICs of itraconazole were 10-fold lower than those of fluconazole. However, there were a few problems peculiar to this drug. Itraconazole is insoluble in water; therefore, the stock solution was made by dissolving the drug in DMSO. This method has been used with other antifungal agents with low water solubilities, such as amphotericin B and ketoconazole (14). Vigorous mixing was necessary to keep the highest concentrations of itraconazole (64 and 32 μg/ml) in solution prior to final inoculation. After incubation for 48 h a small amount of precipitate of itraconazole was noted in the wells with the highest concentration of drug (6.4 and 3.2 μg/ml) in all methods, but it did not interfere with the reading of the MICs. We noticed what appeared to be the reemergence of growth in tubes with the highest concentrations of itraconazole by the NCCLS broth macrodilution method but not the Alamar Blue-modified methods. The mechanism responsible for this observation is not known, but it has been noted previously with other antifungal drugs (3).

The microdilution assay for itraconazole yielded tightly nested results, but the MICs shifted to a higher value compared with those determined by the NCCLS method. Longer periods of incubation (48 instead of 24 h) have been noted to raise the MICs when an Alamar Blue-modified microdilution system is used (10). It is possible that a decrease in incubation time to 24 h may shift the MIC readings toward a closer correlation with those determined by NCCLS methods.

Fluconazole and itraconazole MIC endpoints were more clear-cut by the Alamar Blue-modified methods. For fluconazole, the results obtained by both Alamar Blue-based methods correlated well with those obtained by the NCCLS proposed standard. For itraconazole, the results obtained by the Alamar Blue-modified broth macrodilution method correlated closely with those obtained by the NCCLS method. However, the microdilution technique led to an upward shift in itraconazole MICs, and thus, the discordance between the Alamar Blue-modified microdilution method and the NCCLS standard method was greater. Our data confirm the possible usefulness of this colorimetric microdilution method, but further studies are needed to define the parameters which will allow an even better correlation with the NCCLS standard method.

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