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MINIREVIEW

Susceptibility Testing of Fungi: Current Status of the Standardization Process

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In a previous review of antifungal susceptibility testing (12), several problems were identified that made clinically relevant in vitro tests an illusive goal. Clinical laboratory microbiologists felt little pressure to develop a test that was infrequently requested or that would have little influence on patient management. The few reference laboratories that offered testing derived their methods from their own local experience. This created a diversity of testing methods which produced correspondingly discrepant results (13). Moreover, since virtually no relationship had been established between tests and patient responses, it was not possible to determine which of the methods would best predict benefit from treatment. The National Committee for Clinical Laboratory Standards (NCCLS) established a subcommittee to develop antifungal testing procedures in 1983, but at that time a standardized methodology did not seem possible until some time in the distant future.

During the intervening years, rising numbers of both fungal infections and available antifungal drugs have made both prescribing physicians and clinical pathologists increasingly uncomfortable because of the lack of clinical antifungal tests. Fortunately, with continued support from the NCCLS, the possibilities for meaningful testing have improved considerably. Many people have participated in coordinated efforts to develop standards of potential value to clinical laboratories. Through a series of interlaboratory collaborations, consensus has been developed around a reference testing method for *Candida* species and *Cryptococcus neoformans*, and in 1992 it was published as NCCLS document M27-P (18).

This minireview provides an analysis of how the standard described in NCCLS document M27-P evolved and what specific features of the standard have proved to be important. In addition, information about this standard method's strengths and limitations in identifying resistance for antifungal drugs will be summarized. Finally, potential uses of the method by the research community or prescribing physicians are considered, as is what further work is needed in the decade ahead. The intent here is not to be comprehensive, and more exhaustive reviews of antifungal susceptibility testing have recently been published elsewhere (14, 24).

Development of the NCCLS reference standard. The first activity of the subcommittee for antifungal susceptibility tests was to query NCCLS members regarding their current practices (4). Of those laboratories performing antifungal tests, most were interested in testing *Candida* species and *C. neoformans*, and therefore, as its immediate priority, the subcommittee focused on susceptibility tests for yeasts. Similarly, a broth macrodilution procedure was a reasonable choice since it was the most commonly used test format. However, subcommittee members were uncertain as to how the method would eventually be configured, and therefore, an added attraction of the broth macrodilution method was its flexibility, which would permit analytic studies of nearly any test condition. As things have developed, this flexibility has proven to be critical, especially in studies to determine the best endpoint definition for azole antifungal agents such as fluconazole.

Once the type of test procedure was agreed upon in a general sense, attention was shifted to analyzing a series of specific details of the test configuration. For some details, consensus could be achieved rather easily. For example, a pipetting scheme, previously adopted by the NCCLS for other standards to minimize systematic errors (17), was incorporated for antifungal testing. For other test conditions, collaborative studies demonstrated the suitability of one or another selection. Thus, RPMI 1640, buffered to a pH of 7.0 with morpholinepropanesulfonic acid (MOPS), was selected as the growth medium, 35°C was selected as a practical incubation temperature, and the starting inoculum was prepared by a spectrophotometric method (21, 22).

By 1990, there still remained three important issues that had not yet been resolved. First, whether the incubation time should be 1 or 2 days had not been agreed upon. Second, the definition of the MIC endpoint needed clarification. Third, even though the subcommittee had agreed upon how to prepare the starting inoculum, it had not agreed exactly what the inoculum size should be. In past studies, 10^4 yeast cells per ml had been used, and with this inoculum size, interlaboratory agreement had not always been acceptable (22). Although lowering of the inoculum size might improve reproducibility, testing of a smaller number of yeast cells could theoretically cause problems with yeasts that are heteroresistant, since such resistance might be missed if the inoculum size is smaller than the frequency of resistant clones. This is especially relevant for the susceptibilities of some strains of yeasts for flucytosine (14). These uncertainties led to the formation of a large collaborative study, the results of which were recently published (11). For that study, 13 sites agreed to test the activities of amphotericin B, flucytosine, and ketoconazole against a total of 100 isolates of yeasts. Of these, 85 strains were consecutive, distinct strains of six species which had been isolated from blood cultures at one university medical center, and the other 15 strains were isolates of *C. neoformans* supplied from another institution. In parallel studies, four of the study sites also tested fluconazole (9). Studies were performed by using two different starting inoculum sizes (5×10^4 and 1×10^6 yeast cells per ml), and readings were performed on the first and second days of growth. Each tube was examined for turbid-

TABLE 1. Effect of time of reading, inoculum size, and endpoint definition on agreement among laboratories in antifungal testing[a]

Drug	Endpoint[b]	% Agreement			
		First-day results		Second-day results	
		Low inoculum	High inoculum	Low inoculum	High inoculum
Amphotericin B	MIC-0	85	87	90	87
	MIC-1	81	86	90	89
Flucytosine	MIC-0	66	68	77	65
	MIC-1	57	83	85	79
Ketoconazole	MIC-0	43	48	55	45
	MIC-1	46	42	44	39
	MIC-2	52	69	75	65
Fluconazole	MIC-0	56	49	82	46
	MIC-1	55	73	76	48
	MIC-2	63	79	85	64

[a] Results were adapted from references 11 and 9. For 85 strains of non-*C. neoformans* yeasts whose median results were within the drug dilution range, agreement was calculated as the percentage of observations within twofold of the median. This tabulation of fluconazole results is in conformity with that of Fromtling et al. (11) and differs from the presentation by Espinel-Ingroff et al. (9), in which *C. neoformans* and strains whose median results were off-scale were not excluded.

[b] MIC-0, optically clear; MIC-1, slight haze; MIC-2, substantial reduction in growth.

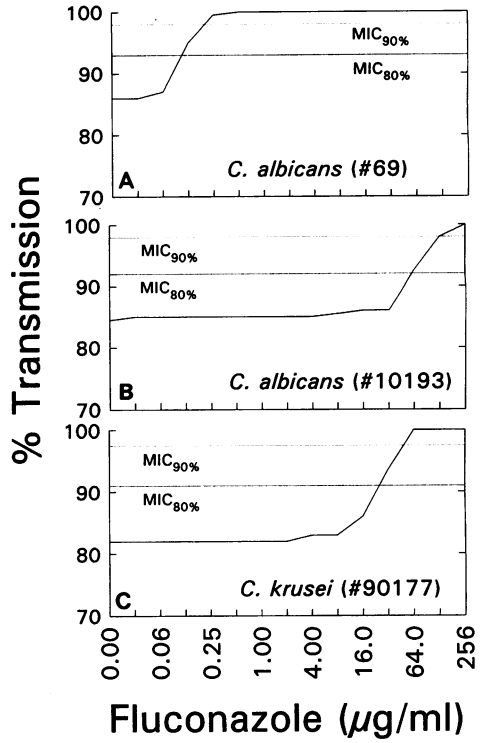

FIG. 1. Effect of increasing concentrations of fluconazole on growth of a typical strain of *C. albicans* (A), a strain isolated from a patient who was failing fluconazole therapy (26) (B), and a strain of *C. krusei* (15) (C). Horizontal dotted lines indicate the turbidity of drug-free control growth diluted with medium in a ratio of 1 to 10 ($MIC_{90\%}$) or 1 to 5 ($MIC_{80\%}$) as labelled.

ity, and its appearance was recorded as a number from 0 (optically clear) to 4+ (no reduction in turbidity compared with that of the drug-free control), thus permitting comparisons of results which utilized various endpoint definitions. Those studies generated about 50,000 results for analysis.

The overall results from those studies are given in Table 1. Isolates whose results clustered above or below the drug dilution range would artificially improve the degree of agreement, and therefore they were excluded. As can be seen, results with the lower starting inoculum read after 2 days generally produced the greatest interlaboratory agreement. Especially important were the differences obtained with different endpoint definitions for flucytosine, ketoconazole, and fluconazole. Use of 0 turbidity as the endpoint criterion resulted in much poorer agreement compared with that resulting from the use of 2+ as the endpoint criterion.

Subsequent to these studies, Espinel-Ingroff et al. (9a) refined the method of defining the partial inhibition by relating it to a standard dilution of the drug-free control tube. By this procedure, the 1+ or 2+ endpoint used by Fromtling et al. (11) is equivalent to a 10-fold dilution ($MIC_{90\%}$) or a 5-fold dilution ($MIC_{80\%}$), respectively. The new definition is less subjective and should reduce interobserver error still further. It should be noted that the subscripted MICs in this context reflect the percent reduction in turbidity and not the percentage of strains inhibited by the specified drug concentration, as it frequently denotes in descriptions of susceptibility test results for antibacterial agents.

That a partial inhibition endpoint such as an $MIC_{80\%}$ would produce better agreement than more stringent endpoints has been a source of considerable confusion for many people. To illustrate this relationship, Fig. 1 depicts the turbidity that developed after incubation of the following three different isolates in different concentrations of fluconazole: a typical strain of *C. albicans* (strain 69), a strain of *C. albicans* isolated from a patient with thrush who had failed fluconazole therapy (strain 10193 [26]), and a strain of *Candida krusei* (strain 90177 [15]), a species that is more difficult to treat effectively with fluconazole (1, 6, 10, 15, 16, 19, 25, 28). Also shown in Fig. 1 are the $MIC_{90\%}$ and $MIC_{80\%}$ criteria as horizontal lines. As can be seen, fluconazole produces a prominent inhibitory effect with all three strains. However, this effect occurs at a lower concentration for the typical *C. albicans* strain compared with that at which it occurs for the two putatively resistant strains. The $MIC_{80\%}$ criterion is sensitive to this difference, producing results of 0.12, 64.0, and 32 μg/ml, respectively, for the three isolates. Corroborating this pattern are studies by Pfaller and Riley (20), which demonstrate that fluconazole affected the sterol and carbohydrate compositions of both *C. albicans* and *C. krusei*, but for *C. krusei*, higher concentrations were required. Although the $MIC_{90\%}$ criterion might also distinguish the first isolate as more susceptible, this criterion is much closer to the slight amount of turbidity often encountered at concentrations of fluconazole above those which produce the prominent inhibitory effect, and consequently, interobserver variability is likely to be greater. As an historical note, this issue was a serious problem early in the development of standards for sulfonamide and other antibacterial agents which did not have sharp endpoints. For those drugs as well, an 80% endpoint was similarly found to be useful for resolving problems of reproducibility (2, 3).

On the basis of these various considerations, consensus was achieved within the subcommittee for a reference standard. Table 2 summarizes the principal elements of that method.

TABLE 2. Major elements of the NCCLS reference procedure for antifungal susceptibility testing

Test parameter	Standard condition
Medium	RPMI 1640
Buffer	MOPS, 0.165 M
pH	7.0
Inoculum preparation	Spectrophotometric comparison with barium sulfate standard
Starting inoculum size	$(1.5 \pm 1.0) \times 10^3$ cells/ml
Drug dilution procedure	Two-stage scheme, minimizing systematic errors
Drug concn tested	Log_2 dilutions above and below 1.0 μg/ml
Incubation temp	35°C
Incubation time	2 days of drug-free control turbidity (2 days for most isolates; 3 days for *C. neoformans*)
Endpoint definition	No visible turbidity for amphotericin B; greater than 80% inhibition for flucytosine, ketoconazole, and fluconazole

Performance of the standard in identifying resistance. Collaborative studies to date have focused on optimizing intralaboratory agreement and have only indirectly addressed the issue of actually detecting strains with unusually high levels of drug resistance. Indeed, it is conceivable that in creating a reproducible method, the sensitivity for outlying strains might have been diminished. The means of discrimination shown in Fig. 1 suggests that this is not the case for fluconazole, and results of initial studies corroborate this impression. For example, Cameron et al. (5) have recently recovered strains of *C. albicans* from patients with or without symptoms of thrush who were or were not receiving an azole antifungal agent and used the reference method to test them for their susceptibilities to fluconazole. Fluconazole MICs for strains from 22 patients who continued to have symptoms despite azole antifungal drug therapy were significantly higher than MICs for strains from 17 patients receiving an azole drug who did not have symptoms. Of additional interest, for strains from patients who had had prior azole therapy, MICs were significantly higher than those for strains from patients who had not had antifungal therapy within the past month. This latter difference is consistent with other observations of Redding et al. (23), who correlated the in vitro susceptibilities of more than 14 isolates collected over a 2-year period from the oral cavity of a single human immunodeficiency virus-infected patient who received multiple treatments with fluconazole for recurrent thrush. During the first nine courses of therapy, $MIC_{80\%}$s shifted upward but remained less than 8.0 μg/ml; responses to therapy were consistently achieved with treatment with 100 mg of fluconazole per day. However, subsequently, the $MIC_{80\%}$s rose and progressively higher doses of fluconazole were needed to produce clinical responses. Similarly, for *Torulopsis glabrata*, considered by many to be refractory to fluconazole treatment (7), MICs were higher than those for most *C. albicans* isolates.

There is less direct in vivo correlation with flucytosine. In the study by Fromtling et al. (11), some of the *C. neoformans* strains were selected because of their previously recognized flucytosine resistance and were identified as resistant by the proposed method. In addition, some of the *Candida* strains demonstrated high-level resistance, as would be expected from previous studies of the prevalence of flucytosine resistance (27).

Although reliable detection of resistance to flucytosine and azole antifungal agents was expected to be the most difficult problem in developing a reference method, an unexpected situation has arisen with amphotericin B. In the most recent collaborative study (11), for all 100 strains of *Candida* species and *C. neoformans*, MICs were 0.5 or 1.0 μg/ml. In particular, none of the *Candida lusitaniae* strains were identified as resistant. Because for some strains of *C. lusitaniae* MICs are greater than those obtained by other methods (8), the *C. lusitaniae* strains were retested by two other laboratories by their own methods (7a, 16a). Both laboratories independently identified two of nine isolates for which MICs were greater than those for the rest of the isolates. These findings raise the issue that the reference method may not detect clinically relevant amphotericin B resistance, and further studies will be needed to determine whether modification of the NCCLS method should be made to exaggerate differences in susceptibility to this agent.

Value of the reference method for research and patient management. The NCCLS reference standard is an important new resource for clinical investigation and, in the foreseeable future, is very likely an important new resource for patient management as well. First and foremost, development of a reference standard makes available a reliable tool which can be linked to clinical outcomes. It is not unreasonable to suppose that a patient whose infecting strain is found to be resistant by this procedure should be more likely to fail therapy, even though many factors in addition to intrinsic drug susceptibility may also be important. It is now possible to test this relationship in a way that will allow comparison among separately conducted studies. As a research tool, this procedure will be especially useful for enhancing controlled clinical trials.

A second major use of the reference method will be to serve as a touchstone for second-generation methods that may better serve practical clinical laboratory situations. Although the broth macrodilution design has facilitated analysis, it is performed manually and is difficult to carry out with large volumes of specimens or in a general laboratory not dedicated to research. Other methods such as broth microdilution, agar diffusion, or proprietary approaches may offer significant advantages in automation, speed, or cost over the reference procedure. Adjustment of these alternative procedures to mimic results obtained by the method described in document M27-P (18) should deliver all of these advantages to clinical laboratories without the likelihood of creating widely divergent results, as was the case in the past (13). It may be that the M-27 reference method will eventually be used almost exclusively to calibrate alternative methods.

Finally, the method described in document M27-P gives laboratories a standardized procedure with which to respond to the requests of treating physicians. The NCCLS currently recommends that tests not be routinely performed for patient care, since the interpretation of results is not yet documented. Results should be viewed as an adjunct to other more direct sources of information, including the efficacy of the agent in experimental infections and the response to therapy by other patients with similar infections. Underscoring the uncertainty about the precise relevance of test results is the subcommittee's hesitancy in offering tentative breakpoints for differentiating susceptible and resistant strains. Nonetheless, even with these substantial limitations, a standardized procedure should resolve many of the discrepan-

cies which until now have existed in results reported from different reference laboratories.

Needs for the decade ahead. The new reference standard, like all NCCLS standards, is an evolving document. With comments and suggestions, it will likely be refined in future versions. For one thing, further validation is needed for strains that are identified as potentially useful for control purposes, and it is possible that other strains might be more suitable for such purposes. For example, no strains of *C. krusei* are included, and a representative strain from this species might be useful for controlling tests with triazole antifungal drugs. The lack of defined breakpoints has already been mentioned. As clinical information becomes available for correlation with in vitro results, it should be possible to add those results to the document as well. Such adjustments are the normal process for NCCLS documents, which continuously respond to new information.

Pharmaceutical manufacturers that are developing antifungal drugs should use document M27-P (18) in all phases of the evaluation procedure. In the past, with antifungal susceptibility testing as an unstandardized field, manufacturers were tacitly exempted from producing clinically applicable in vitro drug profiles for new antifungal drugs. There is now no reason why such information should not be an expected part of the preclinical process, just as it is for antibacterial agents. In applying standard procedures to a new drug, problems in implementing the standard may surface. The earlier that such problems are analyzed, the sooner the reference procedure can be revised to accommodate any drug's special needs.

Finally, with the subcommittee's primary emphasis on *Candida* species and other yeasts, very little progress has yet been made with procedures for testing molds. For molds, attention to special considerations is required, and they offer new challenges to the development of useful testing methodology. How much interspecies variation in drug susceptibilities exists is unknown, but because they produce an increasingly important segment of fungal infections in immunocompromised patients, the question is becoming more and more pressing. As yeast susceptibility testing becomes a more accepted and reliable clinical laboratory procedure, a serious attempt at standardizing mold testing is not an unreasonable expectation in the years ahead.

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