MINIREVIEW

Antifungal Susceptibility Tests

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In comparison with antibacterial agents, drugs for treating systemic fungal infections are newcomers. Amphotericin B, the first major antifungal agent, was released in the late 1950s, whereas sulfonamide was introduced in the 1930s. Not surprisingly, then, this field is about 20 years behind that of antibacterial agents in its growth. However, the increased awareness of fungal infections, especially in immunosuppressed patients, appears to be resulting in a steady proliferation of new and more effective antifungal agents.

As part of these changes there will inevitably be greater demand for in vitro testing. Reliable in vitro testing would facilitate efficient screening for promising compounds. At the clinical level, in vitro tests are expected by clinicians to aid in the optimal treatment of their patients. This mini-review will highlight some of the current issues relating to the reproducibility and relevance of antifungal susceptibility testing as currently practiced. Also to be discussed will be some recent collaborative efforts by the National Committee for Clinical Laboratory Standards to improve test performance.

REPRODUCIBILITY OF IN VITRO ANTIFungal SUSCEPTIBILITY TESTS

There are now two reports indicating the lack of reproducibility among different laboratories performing antifungal susceptibility testing. In the first, Calhoun et al. (6) distributed several strains of Candida albicans to seven reference mycology laboratories. In each laboratory, the isolates were tested for their susceptibility to amphotericin B and flucytosine by the broth dilution method as described in the Manual of Clinical Microbiology (32). Each laboratory repeated the determinations twice on each of 2 separate days. Same-day agreement was virtually perfect (95% of replicates produced identical results), and intralaboratory agreement was acceptable (95% of replicates were within a twofold dilution range). However, variations between the results produced by different laboratories were much greater. Differences with amphotericin B ranged from 8- to 32-fold; with flucytosine, they ranged from 32- to >512-fold.

In the second study, Galgiani et al. (11) selected Candida strains to reflect various susceptibilities to amphotericin B, flucytosine, or ketoconazole. A total of 12 isolates (four for each drug) were distributed to three other laboratories, in which the isolates were tested in a blind fashion on two successive days. For this study, the test methods were not specified; rather, they were selected at the individual laboratories to be those with which each collaborator was most familiar. Even though isolates were tested in a blind fashion, intralaboratory differences among replicates were seldom greater than fourfold for any method. However, interlaboratory differences for each isolate were extreme, ranging from 16- to >50,000-fold.

Although variability of this magnitude is disconcerting, it is entirely consistent with prior studies indicating the dependence of antifungal susceptibility testing results on a variety of test conditions. In general, larger starting inoculum sizes result in higher susceptibility endpoints for flucytosine and several imidazole congeners (3, 4, 12, 13, 25). Medium components may antagonize drug activity (2, 5, 8, 14, 16-19, 25-29, 36). Flucytosine can be antagonized by purines and pyrimidines which may be found in Sabouraud or other complex media and certain buffers such as Tris. It is possible that medium antagonism may account for some of the differences in results when complex media are used for testing ketoconazole. Medium pH can influence antifungal effects (2, 5, 17, 18, 21, 30). Most notable among these is the marked inhibition of ketoconazole below pH 6. In addition, incubation temperature (3, 17, 18, 20) and time of reading results (1, 3, 4, 12, 13, 15, 16, 21, 25, 33, 37) can influence antifungal effects. These types of influences are certainly not unique to the testing of antifungal agents, and similar examples are well recognized for antibacterial agent testing (9). The essential difference between susceptibility testing of fungi and that of bacteria is most likely related to the difference in the amount of effort directed at the problem. To that extent, additional effort should produce antifungal agent testing procedures as reproducible as those currently available for antibacterial agents (22, 34).

CORRELATION OF IN VITRO TEST RESULTS WITH IN VIVO DRUG Efficacy

Despite the lack of interlaboratory agreement, there are now convincing examples of in vitro test results correlating with the treatment of experimental infections in animals. In a study reported by Stillier et al. (35), 40 isolates of C. albicans were selected to reflect different broth dilution MICs of flucytosine. Each isolate was then used separately to infect mice intravenously, and the effect of flucytosine treatment in a murine model was determined. When the in vitro susceptibilities of the different isolates were compared with the in vivo susceptibilities to flucytosine treatment, the correlation was very significant, although additional variables also appeared to account for the residual differences (Fig. 1).

Another set of data has been constructed by John Ryley, who compared in vitro results for 17 antifungal agents, all having a common bis-triazole tertiary alcohol structure, to in vivo efficacy as estimated by a murine model of vaginal candidiasis (F. T. Boyle, J. F. Ryley, and R. G. Wilson, in R. A. Fromplinling, ed., International Telesymposium on Recent Trends in the Discovery, Development and Evaluation of Antifungal Agents, in press). A broth dilution procedure utilizing a turbidometric endpoint produced in vitro 50% inhibitory concentrations ranging from 0.18 to 28 μg/ml against an
The antifungal susceptibility of each isolate of C. albicans. Treatments that resulted in virtually complete control of the vaginal infection ranged from 1 to >100 mg/kg. The correlation between these two indicators was excellent (correlation coefficient, 0.89) (Fig. 2). In the same report, similar data were produced with 43 monotriazoles. Although a significant relationship was noted between the in vitro and in vivo measurements with these congeners as well, the correlation was less exact, possibly the result of differences in metabolic stability among the different agents.

In a third study, O’Day et al. correlated in vitro results with the therapy of experimental fungal keratitis in rabbits (24). Rabbit corneas were abraded, inoculated with a strain of C. albicans, and either treated with topical agents 10 times per day for 2 days or not treated. Efficacy in the animals was measured as the log reduction in CFU of C. albicans in the treated corneas as compared with the control corneas, and MICs were averages of triplicate broth dilution determinations. For amphotericin B, higher MICs for 17 different C. albicans isolates were associated with a lower reduction of colonies than were those obtained for more susceptible isolates (Fig. 3). Similar results were obtained when natamycin was used in place of amphotericin B.

All of these studies involved experimental infections, and similar correlations with clinical results in naturally acquired infections are not yet available to any appreciable extent. The lack of correlations with human treatment may be due to many factors. For one thing, in vitro tests commonly have not been reported in association with clinical results with antifungal agents, in part because of the lack of standard methods. Also, in the past, there has often been difficulty in interpreting the outcome of therapy (7). A third problem has been the presence of confounding circumstances. For example, in one report, an attempt to correlate test results for flucytosine with the results of treatment of cryptococcal meningitis was hampered by the relative susceptibility of all the isolates to amphotericin B (used concurrently with flucytosine) and the immunocompromised status of many of the patients (S. Shadomy, S. Wood-Helie, E. L. Chan, S. Bartivarian, S. White, H. Lang, W. E. Dismukes, and the NIAID Mycoses Study Group, submitted for publication). A final point, perhaps not fully appreciated by medical mycologists, is that a test must show differences among isolates to have any likelihood of predicting possible differences in responses to treatment. For example, test results with 77 strains of Coccidioides immitis produced a single, normally distributed mode for susceptibility to ketoconazole in which the differences among the isolates were no greater than the error of the method (J. N. Galgiani, D. A. Stevens, J. R. Graybill, W. E. Dismukes, G. A. Cloud, and the NIAID Mycoses Study Group, submitted for publication). In this study, therefore, in vitro test results could not be expected to discern responders from nonresponders any more than susceptibility tests of equally susceptible Streptococcus pneumoniae strains will predict responsive or lethal pneumococcal infections (31).

Despite these problems, making correlations between in vitro results and responses to treatment in human infections

**FIG. 1.** Response to flucytosine therapy in mice infected with each of four groups of C. albicans. Susceptibility groups I through IV represent susceptible through resistant isolates. The 50% effective dose (ED50) is defined as the dose required to keep 50% of infected mice alive for a period twice the median duration of survival of untreated mice. The figure is reprinted from reference 35 with permission.

**FIG. 2.** Relationship between in vitro and in vivo activities of 17 bis-triazole tertiary alcohols. Results for which in vivo endpoints were not reached are represented by triangles. IC50, 50% inhibitory concentration. Data are from Boyle et al. (in press).

**FIG. 3.** Correlation between the in vivo efficacy of topical 0.15% amphotericin B and the in vitro MIC for 17 strains of C. albicans in a rabbit model of keratitis. Data are from O’Day et al. (24).
will be critical to establishing the validity of in vitro testing. To this end, the correlations already demonstrated with experimental infections should provide encouragement for attempting analogous correlations in the clinical setting. A recent report has already suggested that efforts in this direction are likely to be forthcoming (29).

CURRENT STRATEGIES FOR IMPROVING TEST PERFORMANCE

In 1982, the National Committee for Clinical Laboratory Standards (NCCLS) established a Subcommittee on Antifungal Susceptibility Testing. One of its first activities was to determine the interest in antifungal susceptibility testing among its membership. From a questionnaire survey, it was apparent that nearly a fifth of its members affiliated with large hospitals were performing susceptibility testing of one sort or another. The species most commonly tested were *C. albicans* and other yeasts, and the drugs most commonly used were amphotericin B, ketoconazole, and fluocytosine. Similar findings in a larger survey were also obtained by the College of American Pathologists (10, 23).

From these reports, it appeared appropriate for the NCCLS to focus initially upon developing methods to be used by reference laboratories based at larger medical centers for the testing of yeasts. Current efforts involve describing a reference method which might improve interlaboratory reproducibility. For such a method, broth dilution procedures will be used, since they are flexible and already in wide use by those already performing antifungal tests and by others for other applications.

One of the most likely sources of variability in testing methods is imprecision in preparing the starting inoculum. This is particularly problematic with fungi because of the difficulty in making even suspensions. Pfaller et al. are currently involved in studies to determine optimal procedures for preparing inocula (M. Pfaller, M. Rinaldi, M. Bartlett, and the NCCLS Subcommittee on Antifungal Susceptibility Testing, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, F72, p. 401).

Media have also been a source of variability in past studies of antifungal agents. In bacterial testing, the selection of Mueller-Hinton medium as the reference medium, while offering many practical advantages regarding availability and cost, has resulted in problems regarding its performance with some antimicrobial agents, such as aminoglycosides and sulfonamides. Since the demand for antifungal agents, although increasing, is still much less than that for antibacterial agents, the NCCLS Subcommittee chose to limit its attention to synthetic media for the initial development of the reference method. Several synthetic media have been used in the past, and studies are under way to compare their performances.

In addition to analyzing individual variables as potential sources of error, it will also be helpful to identify strains that might be useful for quality control purposes. After completing these several subprojects, it should be possible to perform field trials with the resulting method to determine its reproducibility. If this strategy is successful, other procedures which are faster, more convenient, or less expensive than the macrobroth dilution procedure could be standardized against the reference method in future studies.

The testing of molds has not yet been addressed by the NCCLS but is of obvious value. In addition to the problems involved with the testing of yeasts, the testing of molds poses several additional difficulties. Preparing a homogenous starting inoculum from mycelial growth may often be especially difficult. Many of the pathogenic molds are dimorphic, and this fact leads to issues regarding the most appropriate form that should be used for testing. If the tissue phase of the organism is to be tested, growing that form may pose difficulties (e.g., spherules for *C. immitis* or yeasts for *Histoplasma capsulatum, Blastomyces dermatitidis*, or *Sporothrix schenckii*). Under optimal conditions, these organisms may grow slowly relative to bacteria or most yeasts. That some of the “standard” test conditions may conflict with optimal growth conditions only serves to compound the difficulties. Finally, work with many of the pathogenic molds poses major biohazards problems; for these species, careful containment practices are essential for laboratory safety. Because of all of these problems, developing standardized methods for the testing of molds will very likely take much longer than it will for the testing of yeasts.

Until in vitro antifungal susceptibility tests are standardized, several caveats need to be observed in reporting results. First, since endpoint results depend on test conditions, the specific values produced by an unstandardized test will not have the same meaning as will those produced by standardized tests, such as those available for bacteria. In a similar vein, equations such as a “therapeutic index” involving in vitro endpoints and pharmacokinetic measurements are unlikely to yield rational guides to therapy. Second, since methodology is not standard, publications reporting in vitro test results should describe the test methods in detail. For new drugs, it would also be important to explore the effects of variables known to frequently affect test performance with other agents. Third, obtaining endpoints for susceptible isolates is preferable to identifying their susceptibility as being below a certain concentration. Otherwise, it is not possible to determine their relative susceptibilities. This may at times require broad dilution ranges, in which case there is an increased potential for systematic pipetting errors. The chance of pipetting errors can be reduced by adopting the dilution techniques recently described (22). Finally, the reporting susceptibility results without in vivo correlates should be minimized.

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LITERATURE CITED


