Variability of Agar Dilution-Replicator Method of Yeast Susceptibility Testing

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The agar dilution method of in vitro susceptibility testing of fungi was analyzed with a Steers-type inoculum replicator, ten strains, and three drugs. The replicator reproducibly delivered the same inoculum to each series of plates. The minimal inhibitory concentrations of ketoconazole (an imidazole) and 5-fluorocytosine, but not that of the polyene nystatin, were dependent on the initial inoculum size. With the former two drugs, but not with the latter, minimal inhibitory concentrations were highly variable depending on the time of reading. Results with agar and broth dilution methods were divergent, and the differences in minimal inhibitory concentrations were variable in serial comparisons by the two methods. If the agar dilution minimal inhibitory concentrations were determined at first appearance of control growth, a commonly used end point, small variations in the time of reading (as could occur by variation in observer perception of when initial growth appears) induced large variations in the minimal inhibitory concentrations of 5-fluorocytosine and ketoconazole, particularly with rapidly growing strains. Results at 35 and 30°C were similar. The differences in results with the three drugs suggest different mechanisms of action. The variability quantitated with the agar dilution method could result in variability in results between laboratories and even observers in the same laboratory.

A variety of in vitro susceptibility testing methods are available for use with fungi to determine the minimal inhibitory concentration (MIC) of a drug. These include broth dilution, agar dilution, and disk diffusion techniques. Of these, agar dilution is one of the more common methods used in clinical laboratories (3, 4), but the variables inherent in this system have not been clearly defined.

Since the latter method enables MICs for multiple strains to be determined easily and thus offers certain advantages, the variables in this method were studied utilizing a Steers-type inoculum replicator. Ten fungal species were tested for susceptibility against nystatin, 5-fluorocytosine (5-FC), and ketoconazole. Ketoconazole (R41,400) is a water-soluble imidazole, presently under study as an oral antifungal agent (D. Borelli, J. L. Bran, J. Fuentes, E. Leiderman, H. B. Levine, A. Restrepo-M., and D. A. Stevens, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 18th, Atlanta, Ga., Abstr. No. 51, 1978).

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MATERIALS AND METHODS

Ten yeast strains were used: Candida albicans (seven strains), Torulopsis glabrata (one strain), and Candida parapsilosis (two strains). Nine strains were clinical isolates; one was a laboratory-passaged strain of uncertain origin.

These strains were grown in yeast nitrogen base broth overnight at 35°C. The growth was then adjusted to 60% transmittance at 540 nm, using a Coleman Junior spectrophotometer. This reading approximates a concentration of 2 × 10⁶ cells per ml (1). Dilutions resulting in final concentrations of 2 × 10⁵ to 2 × 10² cells per ml were made. Serial dilutions of drug were made in yeast nitrogen base broth and in agar; the final concentrations of nystatin and ketoconazole were 100 to 0.01 μg/ml, and that of 5-FC was 1,000 to 0.02 μg/ml. Preliminary studies showed no antagonism of these drugs by this medium. Ketoconazole as the hydrochloride was dissolved in sterile water before each experiment. 5-FC was dissolved in yeast nitrogen base broth and filter sterilized; it was used freshly prepared or was from thawed samples which had been frozen at −70°C. Nystatin was dissolved in polyethylene glycol, stored at 4°C protected from light, and used within 14 days of dissolution. Preliminary experiments showed no growth inhibition by polyethylene glycol at the concentrations present in the final nystatin solutions used. Broth dilution MIC determination and determination of the drug
concentration resulting in 50% inhibition (IC50, turbidimetric criteria) were performed by methods previously described (1). For the agar plate method, 0.5 ml of each dilution of yeast in broth was placed in a separate well of a seed block of a Steers-type replicator (Melrose Machine Shop, Woodlyn, Pa.). Triplicates of agar plates of each drug concentration were inoculated by using the replicator to contact the prongs and agar surface.

It was necessary to first determine that the replicator apparatus could reproducibly deliver the same inoculum after the repeated dipping in the wells needed to inoculate several plates. Inocula of one Candida strain were prepared at $2 \times 10^8$ and $2 \times 10^9$ cells per ml as described, the seed block was filled, and 25 yeast nitrogen base plates were serially inoculated. To assay the delivery quantitatively, the contact point was removed from each plate by undercutting with a sterile surgical scalpel. The fragment of agar removed was then placed in 10 ml of yeast nitrogen base agar in a tube at 56°C. It was necessary to undercut as small a volume of solid agar as possible to ensure complete liquefication in the diluent agar. The tube contents were then blended in a Vortex mixer and poured into petri dishes, which were then incubated at 35°C. The resultant colonies were counted at 24 h.

In all experiments, control plates containing only yeast nitrogen base were inoculated at the beginning and end of each series of drug concentrations. This provided a control in each run for changes in inoculation by the replicator, uneven mixing of the inoculum in the wells during inoculation, or loss of viability of portions of the inoculum during an experiment.

Broth dilution end points were determined at 48 h and then daily for 7 days, with the tubes being shaken before each reading. Agar plates were also examined daily; the MIC was defined as the lowest drug concentration at which no growth occurred.

In one experiment, the agar plates were inspected frequently, beginning during the first 24 h after inoculation, in an effort to determine the MIC at the time when growth was first apparent on the control agar plate. This point for MIC determination is often used in clinical laboratories. Further readings were made at frequent intervals after the initial reading to ascertain changes in MIC over the first 56 h (3). All readings in an experiment were performed by the same observer.

### RESULTS

**Reproducibility of replicator delivery.** An experiment was performed with two inoculum sizes, by the method described, to study variability in replicator delivery from plate to plate and also possible differences induced by different depths of fluid in the wells after repeated sampling. Without refilling the wells, there were minute differences in inoculum delivery between plates 1 and 25 in a series. For example, from an inoculum of $2 \times 10^8$ organisms per ml, 16 colony-forming units were delivered to plate 1 and 15 colony-forming units to plate 25; with an inoculum of $2 \times 10^9$ organisms per ml, the counts were 5 and 4 colony-forming units, respectively.

**Inoculum dependence of MIC by agar dilution method for some drugs.** The effect of inoculum size on the MIC when determined by the agar dilution method was examined with six strains of yeasts. For these studies, the MIC was determined, by the criteria described, at 48 h. The inoculum sizes used represent a range of those in common use.

In studies with ketoconazole (Table 1), the effect of inoculum size was large with three strains. With these, the strains were interpreted as highly susceptible when $10^5$ colony-forming units/ml was used as the inoculum but highly resistant when $10^4$ colony-forming units/ml was used. Some effect of inoculum size was apparent with all strains tested, although the effect was modest with one strain which had a long generation time.

Similar studies were performed with nystatin (Table 1). No effect of inoculum size was noted.

Results with 5-FC were similar to those with ketoconazole. However, the MICs for five of six strains were only 0.25 μg/ml even with the highest inoculum tested ($10^6$/ml). With three of these five strains the variation from $10^5$ to $10^6$/ml was only four to eight-fold. The MIC for the sixth

| Yeast strain          | Ketoconazole | Nystatin |
|-----------------------|--------------|----------|
|                       | 10⁵          | 10⁴      | 10³      | 10¹          | 10⁴      | 10³      |
| C. albicans St        | NT¹          | 0.05     | 100      | 100          | 6.25     | 6.25     | 6.25     |
| C. albicans Lu        | NT¹          | 0.05     | 100      | 100          | 6.25     | 6.25     | 6.25     |
| C. albicans Ba        | NT¹          | 0.05     | 100      | 100          | 12.5     | 12.5     | 12.5     |
| T. glabrata Wa        | 25           | 50       | 100      | 100          | 6.25     | 6.25     | 6.25     |
| C. parapsilosis Me    | NT¹          | NT       | NT       | NT           | 6.25     | 6.25     | 6.25     |
| C. parapsilosis Wi    | NT¹          | NT       | NT       | NT           | 6.25     | 6.25     | 6.25     |

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¹ Determinations were performed at 48 h.
² Inoculum size, number of organisms per milliliter.
³ NT, Not tested.
strain was 512 μg/ml when tested with an inoculum of 10^9 organisms per ml; the MIC was 10,000-fold lower when the inoculum was 10^3 organisms per ml.

Variation of agar dilution MIC with time of reading. Six strains were used with each drug for studies of the effect of time of reading on observed MICs, and as a result of the preceding experiments, several inoculum sizes were studied. Data obtained with C. albicans strain St were chosen to display the results of these tests.

With ketoconazole, and with several inocula, the MIC increased as the incubation time was prolonged (Table 2). This occurred with both agar dilution and broth dilution methods.

In contrast, the MIC of nystatin for these strains was relatively constant over time (Table 2). The modest increase observed at 48 h is consistent with the observed relative instability of nystatin and other polyenes at 35°C for prolonged periods.

Results with 5-FC again resembled those with ketoconazole. With five of six strains, however, the absolute variation in MICs was less marked since the MICs were ≤2 μg/ml with all inocula tested and with readings made during 48 to 168 h. With these, the MIC varied ≤fourfold between 48 and 168 h (five organisms, three inocula each), with three exceptions. With two, the variation was indeterminate because the initial MIC at 10^5/ml was below the range of dilutions tested, and in the other the variation was eightfold. The MIC for one strain was ≥64 μg/ml with all inocula at 168 h, when readings were made and the variation between 48 and 168 h for this "resistant" strain was 1,000-fold with two of the inocula tested.

The data for the strains tested can be shown by comparing the percentage of strains showing a ≥fourfold increase in the MICs of nystatin and ketoconazole at different time periods during incubation compared to the 48-h readings (Table 3). The effect of time on the MIC of ketoconazole in contrast to that of nystatin is readily apparent from the data presented.

Comparison of broth and agar dilution MICs. A comparison of two commonly used methods, agar dilution and broth dilution, was performed with several organisms, several inoculum sizes, and several times of reading, using ketoconazole and nystatin as previously shown to be inoculum- and time-dependent and inoculum- and time-independent drugs, respectively. The results can be displayed as the ratio of MIC in broth to that in agar (Table 4). There was no concordance between the two methods; the broth dilution method gave a consistently higher

### Table 2. Comparison of agar and broth dilution susceptibility testing: effect of time of reading and inoculum size

| Method       | Drug       | Inoculum size (organisms/ml) | MIC (μg/ml) at time of reading |
|--------------|------------|------------------------------|-------------------------------|
|              |            | 24 h | 48 h | 72 h | 96 h | 120 h | 168 h |
| Agar dilution| Ketoconazole| 10^2  | 0.05 | 25   | 50   | 100   |
|              |            | 10^2  | 0.05 | 25   | 50   | 100   |
|              |            | 10^4  | 100  | >100 | >100 | >100  |
|              |            | 10^6  | 100  | >100 | >100 | >100  |
| Nystatin     | 10^2       | 3.1  | 3.1  | 3.1  | 3.1  | 3.1   |
|              | 10^4       | 3.1  | 6.3  | 6.3  | 6.3  | 6.3   |
|              | 10^6       | 3.1  | 6.3  | 6.3  | 6.3  | 6.3   |
| Broth dilution| Ketoconazole| 10^2  | 10   | 10   | 25   | >100  |
|              |            | 10^4  | 50   | >100 | >100 | >100  |
|              |            | 10^6  | >100 | >100 | >100 | >100  |
| Nystatin     | 10^2       | 12.5 | 25   | 25   | 25   | 25    |
|              | 10^4       | 12.5 | 25   | 50   | 50   | 50    |
|              | 10^6       | 12.5 | 50   | 50   | 50   | 50    |

* C. albicans St was the test strain used.

### Table 3. Serial determinations of susceptibility by agar dilution

| Drug       | No. of strains showing ≥4-fold change in MIC/total tested (compared to 48-h reading) |
|------------|----------------------------------------------------------------------------------|
|            | 72 h | 96 h | 120 h | 144 h |
| Ketoconazole| 5/6  | NT   | 5/6   | 6/6   |
| Nystatin   | 0/6  | 0/6  | 1/6   | 1/6   |

* Six yeast strains were tested, with an initial inoculum of 10^5 organisms per ml. NT, Not tested.
end point. This was true with all inoculum sizes examined, as shown with ketoconazole. Some variation in this ratio between inoculum sizes is noted.

With nystatin, time induced less change in the MIC, as Table 4 shows.

**Effect of determining results at first control growth** and effect of small variations in time of initial reading on agar dilution MIC. As stated previously, agar dilution susceptibility testing results are often read at the time of first appearance of control growth. Studies were made at this point in time, serial readings were made at later periods, and comparisons were made with drug concentrations resulting in 50% inhibition (IC$_{1/2}$).

Results with two representative strains and ketoconazole are shown in Table 5. No growth was detected on the control plates for 16 h. The MIC at first reading did not correlate with the 50% inhibitory concentration results. Small variations in time of reading could induce large variations in MIC results with some inoculum sizes, particularly with the faster-growing strains (e.g., C. albicans St).

**Effects of temperature on MIC determinations.** These studies were repeated at 30°C with ketoconazole and nystatin, with several strains and several inoculum sizes, and the results were compared with those at 35°C. Some laboratories use the lower temperature for susceptibility testing.

With six strains and ketoconazole, variation in the MIC, as measured at 30°C by the agar dilution method and dependent on inoculum size and time of reading, showed only minor differences from the data obtained from studies done at 35°C. Inoculum dependence of the MIC result was less prominent at the initial reading (17.5 h). For example, inocula of 10$^4$ and 10$^5$ cells per ml gave identical results, whereas only the MIC with an inoculum of 10$^5$/ml showed the marked

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**Table 4. Ratio of MIC determined by the broth dilution method to that determined by the agar dilution method**

| Drug                  | Strain          | MIC ratio (µg/ml) at inoculum size (organisms/ml) of: |
|-----------------------|-----------------|---------------------------------------------------|
|                       |                 | 10$^3$    | 10$^4$    | 10$^5$    |
| Ketoconazole 48-h reading | C. albicans St | >10       | 8          | 8          |
|                       | T. glabrata Wa | 2         | 2          | 8          |
|                       | C. albicans Lu | >10       | 8          | >10        |
|                       | C. albicans Ba | >10       | >10        | >10        |
| Nystatin, effects of time and inoculum size | T. glabrata Wa | 48 h  | 4          | 4          |
|                       |                 | 96 h  | 4          | 4          |
|                       |                 | 120 h | 4          | 4          |
|                       | C. albicans Lu | 24 h  | 16         | 16         |
|                       |                 | 48 h  | 8          | 8          |
|                       |                 | 96 h  | 8          | 8          |
|                       |                 | 120 h | 8          | 8          |

* Time of reading.

**Table 5. Variation of agar dilution MIC in multiple readings at initial appearance of control growth**

| Yeast strain | Time of reading (h) | MIC (µg/ml) of ketoconazole at inoculum size (organisms/ml) of: |
|--------------|---------------------|---------------------------------------------------------------|
|              |                     | 10$^3$    | 10$^4$    | 10$^5$    |
| C. albicans St | 16                   | —         | 5          | 5          |
|              | 23                   | 1.0       | 25         |
|              | 27                   | 1.0       | 50         |
|              | 56                   | 50        | 100        |
| C. albicans Ba | 16                   | —         | —          |
|              | 23                   | —         | 2.5        |
|              | 27                   | 1.0       | 5          |
|              | 56                   | 2.5       | 5          |

* — No growth on control plate.
divergence seen at 35°C (e.g., >200-fold less for five of six strains). For 24-h and subsequent readings the results were indistinguishable from those with studies done at 35°C. There was no difference in the rate or magnitude of change of MIC over time at the two temperature settings. The lack of correlation of results with either the broth dilution-visual end point or the turbidimetric method was again noted at 30°C. Studies with nystatin gave results identical to those obtained at 35°C.

In the course of these studies we also showed that the variables described in the broth dilution method at 37°C with miconazole and amphotericin B (1) were also present in the broth dilution method with ketoconazole and nystatin at 35°C.

**DISCUSSION**

These results demonstrate several key variables in the agar dilution method of susceptibility testing of yeast not previously quantitated with these organisms.

MICs of ketoconazole and 5-FC, but not that of nystatin, were dependent on inoculum size. Variability of MIC results with time of incubation was seen with the former two drugs but not with nystatin. With ketoconazole and nystatin, there was consistently poor concordance in repeated serial determinations between agar dilution susceptibility test results and broth dilution susceptibility test results. If the agar dilution MICs were determined at first appearance of control growth, as is commonly done, small variations in the time of reading (as could occur by variation in observer perception of when initial growth appears) induced large MIC variations, particularly with rapidly growing strains.

Variability of results was generally greater with ketoconazole than with 5-FC. Against most of the strains used, MICs of 5-FC were consistently well below achievable plasma concentrations, whereas the variability of MICs seen with ketoconazole resulted in a range sufficiently large to encompass concentrations well below those achievable in plasma and concentrations well above those achievable in plasma (Brass and Stevens, unpublished data). These modest differences in results between these two drugs may be due to the exquisite sensitivity of five of the six species used in tests with 5-FC. Growth could occur (the total mass dependent on inoculum size), but the rate could be so slow that at some concentrations it would not reach the visual threshold over the period of observation. Alternatively, at some drug concentrations growth in agar may plateau at concentrations of organisms which are below the visual threshold. The differences in results between 5-FC and ketoconazole may disappear if organisms with a broader distribution of susceptibility to 5-FC are used.

Thus, with some drugs, MIC results by the agar dilution method are dependent on several variables, and one's choices in controlling such variables will arbitrarily determine the final MIC result. This variability would result in considerable discrepancy in results between different laboratories and, with some variables, even between observers in the same laboratory. These variables are also present in the usual method of determining MIC results by dilution in liquid medium and by use of a visual end point, as previously described (1). A spectrophotometric method, which determines end point as a function of control growth, was shown to be free of dependence on inoculum size, and if read during log-phase growth in the controls, the end point did not vary during this interval (1).

Major differences were seen between 5-FC and ketoconazole on the one hand, which were dependent on all the variables indicated, and nystatin on the other hand, which was not dependent on these variables. Similar results were reported previously with the broth dilution method for 5-FC and miconazole (like ketoconazole, an imidazole drug), on the one hand, and amphotericin B (like nystatin, a polyene drug) on the other (2). It was shown in that system that the onset of growth inhibition by the polyene was uniform and independent of generation time of the fungus, in contrast to the other drugs. We hypothesize that these differences reflect mechanism of action, with 5-FC and imidazoles principally acting by metabolic inhibition and requiring actively growing organisms and polyenes acting by membrane binding, presumably with an irreversible effect at any phase of growth.

If correlations are to emerge between in vitro susceptibility and clinical results with antifungal chemotherapy, in vitro methods with controlled variables are undesirable. We have found the spectrophotometric method (1) easy to perform, and due to its independence of growth characteristics and inoculum size of the organism, we suggest that this method should be used widely for in vitro susceptibility testing. If other methods are used, such as the agar dilution-replicator method, attempts should be made to control all variables (e.g., inoculum size) possible.

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