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Antimicrobial Susceptibility Testing of Six Clinical Isolates of *Aspergillus*

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Several different methods of performing susceptibility tests on six clinical isolates of *Aspergillus* are described. Some of the conditions that affected the level of susceptibility to drugs were: the type of media used, temperature and time of incubation, and the initial inoculum size. For amphotericin B susceptibility testing, the effectiveness of the polyene antibiotic as measured by visual growth was equivalent to the effectiveness as measured by inhibition of ribonucleic acid synthesis and dry-weight increase. For 5-fluorocytosine and rifampin, the visual-turbidity method gave minimum inhibitory concentrations that were much higher than those determined by effects on ribonucleic acid synthesis and dry weight. The reason for these discrepancies in susceptibility testing with 5-fluorocytosine and rifampin are unknown. We conclude that the most relevant test of this fungus to antifungal agents will have to be determined by the correlation of in vitro data with animal experiments and clinical results.

Unlike the case with bacteria, there is no standard universally accepted method of antimicrobial susceptibility testing for filamentous fungi. There are several reasons for this, the most important being: fungus infections are uncommon compared with bacteria, and experience with fungal susceptibility testing is limited; the variability in morphology, growth rate, and optimal conditions of growth of fungi have made standardization difficult; and the instability and variable solubility of the most important antifungal agents complicate the tests (8).

We have been screening clinical isolates of *Aspergillus* for their susceptibility to different antimicrobial agents. Besides the usual problems regarding the choice of medium, inoculum size, and method of susceptibility testing, the most appropriate temperature and length of incubation for the susceptibility tests are not known. In addition, it is not clear whether the susceptibility tests should be performed on conidiospores, recently germinated spores, or mature mycelium. Although the number of reports on susceptibility testing of *Aspergillus* is small, there are contradictory recommendations for each of the above variables (5, 6, 17).

This report describes our efforts to evaluate all of the above alternatives in susceptibility testing of several isolates of *Aspergillus*. In addition, we have compared the results of the tube dilution turbidity method, which is one of the most accepted methods of determining drug susceptibility, with several other methods we have used.

MATERIALS AND METHODS

Chemicals. Amphotericin B (AmB), in the form of Fungizone, was purchased from E. R. Squibb & Sons Inc., Princeton, N.J. It was dissolved in sterile water before use. Rifampin was obtained from Dow Chemical Co., Zionsville, Ind. Eight milligrams of rifampin powder was dissolved in 0.5 ml of absolute ethanol plus 7.5 ml of distilled water. 5-Fluorocytosine (5FC) was obtained from Hoffmann-La Roche, Nutley, N.J., and was dissolved in distilled water. [5-^{3}H]uridine (specific activity, 8 Ci/mmol) was purchased from Schwarz/Mann, Orangeburg, N.J.

Organisms. The three isolates of *Aspergillus fumigatus* and three isolates of *Aspergillus flavus* used were obtained from human clinical specimens by the mycology laboratory of Barnes Hospital, St. Louis, Mo. The organisms were grown on Sabouraud dextrose agar at room temperature and transferred weekly.

Susceptibility tests. All of the susceptibility tests were done in duplicate at least three times, and in all cases there was no significant variation within each experimental method. Conidiospores were harvested from 7-day-old cultures by flooding the surface growth with buffered saline and shaking the tubes to dislodge the spores. The spore suspensions were then agitated with glass beads and resuspended in 50-ml Erlenmeyer flasks in 2× Salvin liquid medium (14), Czapek-Dox liquid medium (4), or yeast nitrogen base (YNB) (17) at a concentration of either 2×10^5 or 2×10^3 spores per ml. Some experiments were done using 50% serum and 5% dextrose in water or YNB medium. The serum was pooled human serum previously heated to 56 C for 30 min to inactivate complement. The spore number was estimated by hemocytometer counts and confirmed by viable colony counts. One-milliliter samples were pipetted into capped tubes, and an 18-ml

sample of spore suspension was left in each of the Erlenmeyer flasks.

To determine the effects of temperature of incubation on antibiotic susceptibility of ungerminated conidiospores, test drugs were added immediately to duplicate sets of tubes and flasks; one set was incubated at 22 C for 24 h, and the other was incubated at 37 C. The effect of temperature of incubation on the antibiotic susceptibility of germinated spores was determined by allowing duplicate sets of tubes and flasks of conidiospore suspensions to germinate in the absence of drugs at an incubation temperature of 22 C for 22 h or 37 C for 8 to 10 h. Germination was documented by microscopic observation for germ tube formation. The appropriate drug concentrations were then added, and incubations continued for 24 h either at 22 or 37 C.

At the end of 24 h of incubation with the drugs, the cultures were processed in the following manner.

(i) The tubes were read visually for turbidity and graded 0 to 4+. The minimum inhibitory concentration (MIC) of each drug was defined as that concentration which caused complete growth inhibition or no detectable visual turbidity in the tube. Subcultures of the clear tubes were done, and the absence of growth of fungi on plates determined the minimum fungicidal concentration.

(ii) After the turbidity readings, 0.5 μCi of [^{3}H]uridine per ml was added to each tube, and the cultures were reincubated at the original temperatures for 1 h. An equal volume of 10% trichloroacetic acid was added to each tube, and the tubes were cooled in ice for 30 min and then filtered on 2.4-cm glass-fiber filters (Reeve-Angel, Clifton, N.J.). The filters were placed in counting vials, dried under a heat lamp, and counted in Bray solution on a liquid scintillation counter.

(iii) The cultures in the Erlenmeyer flasks (18 ml) were cooled and 2 ml of 50% trichloroacetic acid was added. They were then filtered on preweighed glass filters, dried in an oven, and weighed in tared vials on a Mettler H20T balance.

After the weights were noted, 2 ml of 1 N NaOH was added to each vial and the vials were incubated at 37 C for 1 h. The hydrolysates were centrifuged, and ribonucleic acid (RNA) was determined on the supernatant fluid by a modification of the Schmidt-Thannhauser procedure (7).

Polyacrylamide gel electrophoresis of extracted RNA. Germinated conidiospores incubated in Falcon flasks with [^{3}H]uridine (2 μCi/ml) for 8 h at 37 C were harvested by centrifugation and homogenized with 2 volumes of glass beads (0.11-mm diameter) in a cell homogenizer (Braun model MSK, Bronwill 2876) cooled with liquid CO_2, and the total RNA was extracted by the phenol method described by Penman (12).

Acrylamide gel electrophoresis of the RNA was carried out in 2.7% acrylamide for 3 h after a prerun of 1 h. The gels were run in 10-cm glass columns at room temperature with a constant current at 5 mA/gel. Bromophenol blue was used as a marker for the front, and ^{14}C-labeled 18*S* and 28*S* RNA markers were run with each RNA. After this the gels were frozen in dry ice; 1.5-mm slices were incubated in scintillation vials with 0.3 ml of concentrated NH_4OH for 5 h at room temperature. Then 5 ml of Bray solution was added and the samples were counted in a liquid scintillation counter.

RESULTS

Of the radioactive precursors tested, [^{3}H]uridine and [^{3}H]guanine had the highest levels of incorporation into the trichloroacetic acid-precipitable fractions of *Aspergillus* (Fig. 1). [^{3}H]thymidine, [^{3}H]uracil, and [^{3}H]leucine were not incorporated. The level of incorpora-

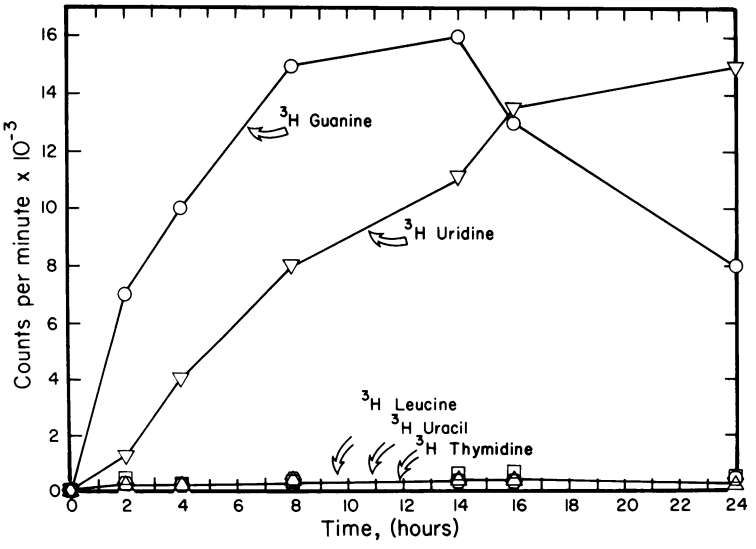

FIG. 1. *Incorporation of [^{3}H]uridine (▽), [^{3}H]guanine (○), [^{3}H]uracil (□), [^{3}H]thymidine (△), and [^{3}H]leucine (◇) into trichloroacetic acid-precipitable fractions of A. fumigatus 1.*

tion of [^{3}H]uridine into acid-insoluble fractions of whole cells was equal to the amount incorporated into acid-insoluble fractions of broken cells. Fifty to sixty percent of the incorporated label could be recovered in the RNA fractionated on polyacrylamide gels, with most of the label going into ribosomal RNA and its precursors (data not shown). Therefore, all measurements of RNA synthesis by the radioactive method were done by determining [^{3}H]uridine incorporation into acid-insoluble fractions of whole cells.

The visual-turbidity susceptibility tests were read after 24 h of incubation because this was the shortest period of incubation that allowed visible growth in the control tubes. Because there were no differences in the MICs obtained by using germinated or ungerminated spores in the initial inoculum, ungerminated spores were used in all of the experiments.

Table 1 shows the MICs for each of the three agents tested against one isolate each of *A. fumigatus* and *A. flavus* in several different incubation media. The MIC for AmB was much lower in Salvin or Czapek-Dox medium than in YNB. When the medium contained 50% serum, the MIC was closer to the results obtained with Salvin and Czapek-Dox media than the results with YNB. The MICs for 5FC were also markedly different in the different media, but in this case they were much lower in YNB than in the other media. The MIC for rifampin was the same in all of the media tested. Essentially the same pattern of results of susceptibility testing was seen with the other strains of *Aspergillus*. Because the AmB and 5FC MICs in Salvin medium were closest to the results obtained with 50% serum, we performed all of our subsequent susceptibility tests in Salvin medium.

Table 2 lists the MICs for each of the three agents tested against all of the clinical isolates of *Aspergillus*. The MICs of AmB against the three isolates of *A. fumigatus* were significantly less than those against *A. flavus*. The MICs for 5FC and rifampin were very high for all six isolates. In every case the MICs for all three agents were markedly lower at an incubation temperature of 25 C than they were at 37 C.

The initial inocula also influenced the MICs. Much lower MICs were seen with an initial inoculum of 10^3 organisms/ml than with 10^5 organisms/ml (Tables 2 and 3).

The dose response curves to each of the drugs were very similar when determined by [^{3}H]uridine incorporation into RNA, biochemical determination of the amount of RNA, and measurement of dry weight. Examples of the dose response curves are presented for only one of the strains of *A. fumigatus* and *A. flavus* tested, but the same results were seen with all of the other strains in all of the media used.

There was excellent agreement between the MICs of AmB as determined by visual turbidity

TABLE 1. *MICs of various drugs in different media*[a]

Medium	MIC (μg/ml)					
	A. fumigatus 3			*A. flavus 2*		
	AmB	5FC	Rifampin	AmB	5FC	Rifampin
Salvin	1.0	>100	1,000	5.0	>100	1,000
Czapek-Dox	1.0	>100	1,000	10.0	>100	1,000
50% serum + 50% distilled water	5.0	>100	1,000	5.0	>100	1,000
50% serum + 50% YNB	5.0	>100	1,000	5.0	>100	1,000
YNB	50.0	0.1	1,000	>50.0	1.0	1,000

[a] Incubation was at 37 C.

TABLE 2. *MICs of the drugs at different temperatures of incubation*

Organism	MIC (μg/ml)					
	AmB		5FC		Rifampin	
	25 C	37 C	25 C	37 C	25 C	37 C
A. fumigatus 1	0.1	0.5	5	100	25	1,000
A. fumigatus 2	0.1	0.5	25	250	50	1,000
A. fumigatus 3	0.1	1.0	25	200	25	1,000
A. flavus 1	0.2	2.5	10	200	500	1,000
A. flavus 2	0.5	4.0	20	250	100	1,000
A. flavus 3	0.2	2.0	50	500	50	1,000

and measures of RNA synthesis and increase in dry weight (Fig. 2). In all organisms, the MICs for AmB as determined by turbidity corresponded closely to the concentrations of AmB that resulted in a 50% inhibition of RNA synthesis and dry-weight increase. For 5FC (Fig. 3) and rifampin (Fig. 4), however, there were marked discrepancies between the values. In every case, the levels of drugs that resulted in a 50% inhibition of RNA synthesis and dry-weight levels were much lower than the MICs as determined by turbidity.

TABLE 3. *MICs of the drugs at initial inocula of 10^3 organisms/ml*[a]

Organisms	MIC (μg/ml)		
	AmB	5FC	Rifampin
A. fumigatus 1	0.1	5	100
A. fumigatus 2	.05	5	100
A. fumigatus 3	0.1	5	100
A. flavus 1	0.5	10	100
A. flavus 2	0.5	5	100
A. flavus 3	0.25	10	100

[a] Incubation was at 37 C.

DISCUSSION

Despite the increasing frequency of human infections caused by species of *Aspergillus*, there is a paucity of data concerning the susceptibility of this fungus to AmB and 5FC. In general, most of the studies have involved only a few isolates and the susceptibility tests have been performed by a variety of methods (6, 5, 17). MICs of AmB greater than 10 μg/ml have been found by some workers (6, 10), whereas others (5, 15) have reported inhibitory levels of less than 1 μg/ml. It is likely that differences in the methodology used are important determinants of the variability in AmB susceptibilities found in the literature.

Even less information is available on the sus-

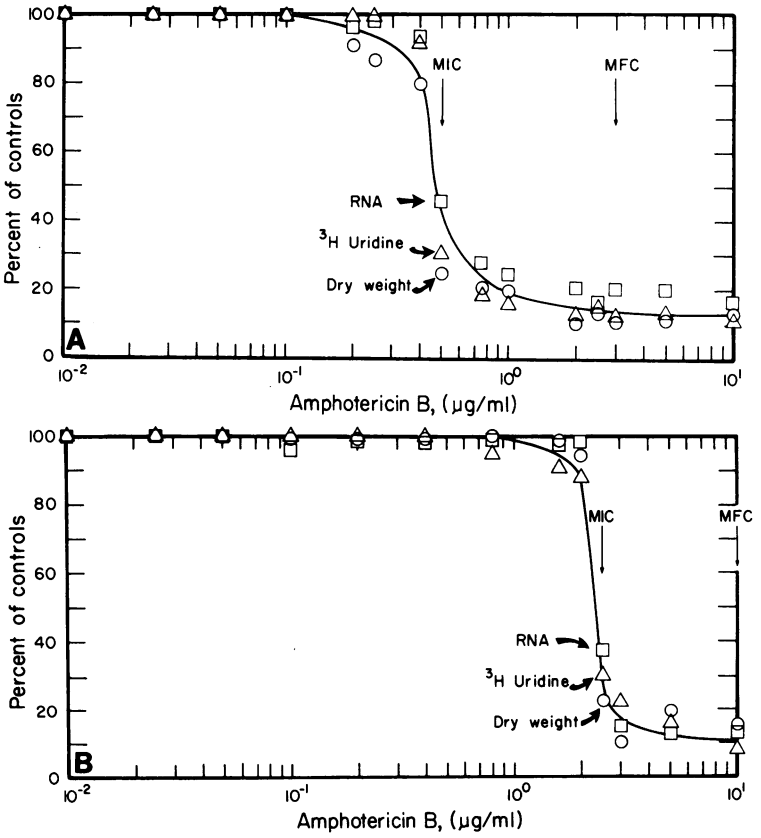

FIG. 2. *Dose responses in A. fumigatus 1 (A) and A. flavus 1 (B) of RNA determinations (□), dry-weight determinations (○), and [^{3}H]uridine incorporation into RNA to increasing concentrations of amphotericin B. The MICs and minimum fungicidal concentrations (MFCs) as measured by visual turbidity are marked by the arrows. Incubation was at 37 C.*

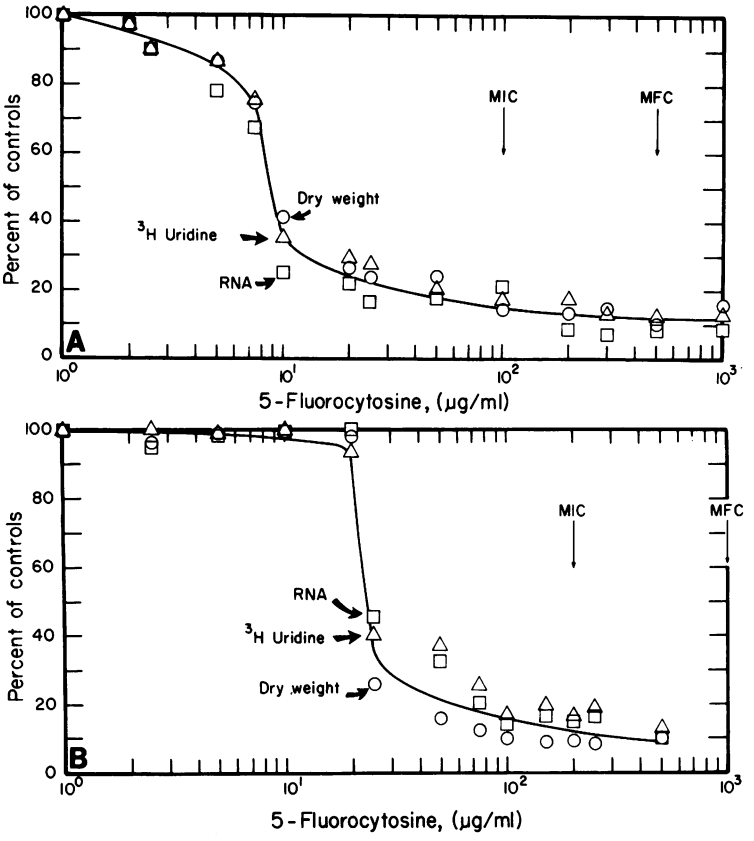

FIG. 3. *Dose responses in A. fumigatus 1 (A) and A. flavus 1 (B) to increasing concentrations of 5FC. Incubation was at 37 C.*

ceptibility of *Aspergillus* to 5FC. Although there have been reports of clinically successful treatment of *Aspergillus* infections with 5FC, only three of seven isolates of *Aspergillus* were inhibited by clinically achievable levels of 5FC in one report (16), and in another the MIC for 5FC against a clinical isolate of *Aspergillus* was greater than 500 μg/ml (6). Recently, a new method for susceptibility testing of fungi has been described (17). Using this method, five of seven isolates of *Aspergillus* were found to be susceptible to less than 0.5 μg of 5FC per ml. However, in that report the temperature of incubation was 30 C and the initial inoculum, which consisted of a mixture of ungerminated spores and mycelial fragments, was not carefully quantitated in terms of cell number.

Our data emphasize that the type of medium used, the temperature of incubation, and the initial inoculum markedly affected susceptibility levels. Salvin medium is a semisynthetic medium, and MIC determinations done in it were markedly different from the results obtained when the synthetic medium YNB was used. It is difficult to explain the different results with the two media on the basis of one being semisynthetic (Salvin) and the other a totally defined synthetic medium (YNB), because Czapek-Dox medium is also synthetic and the results obtained by using it were similar to those obtained by using Salvin medium. We feel that the most clinically relevant medium was the one containing 50% serum, and because these results obtained with serum were similar to those obtained with Salvin and Czapek-Dox media, we used Salvin medium for all our experiments.

Lowering the temperature of incubation and decreasing the inoculum markedly reduced the MICs. Here also, it is not clear which conditions yield the most clinically relevant data. It seemed to us again that the in vitro conditions of incubation should reflect, as closely as possible, in vivo situations; therefore, a 37 C incubation temperature would be most reasonable. The choice of inoculum size is arbitrary. We used an initial inoculum of 10^5 organisms/ml because this number grew well enough so that

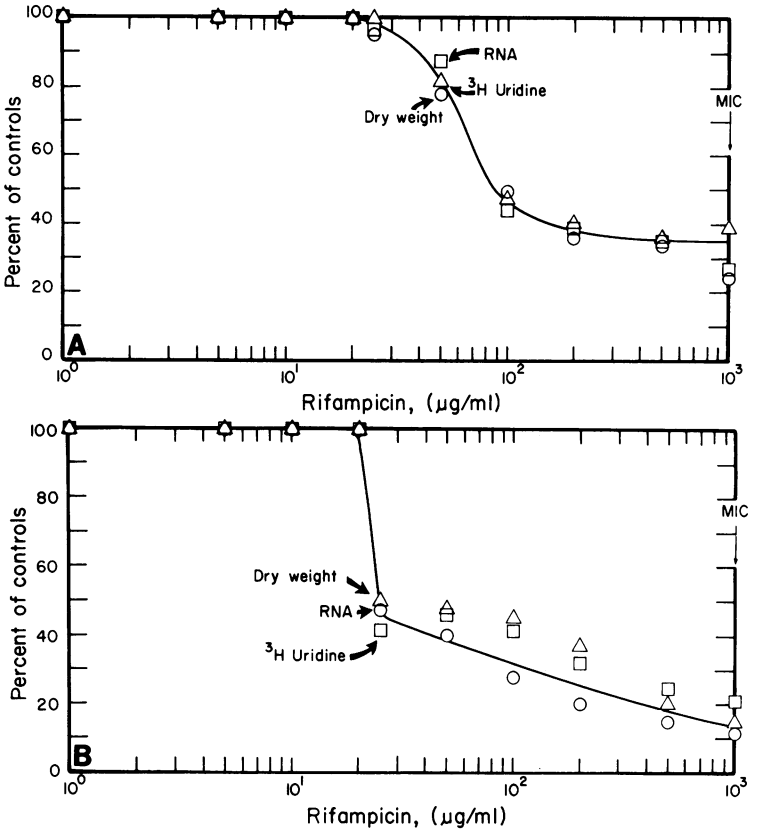

FIG. 4. *Dose responses in A. fumgiatus 1 (A) and A. flavus 1 (B) to increasing concentrations of rifampin. Incubation was at 37 C.*

radioactive incorporation was efficient and visual turbidity could be read at 24 h. The susceptibility levels were the same in our experiments when germinated spores or ungerminated spores were used as the inoculum.

Another important determinant of the MIC is the length of incubation. AmB is unstable in Salvin medium, with a half-life of about 18 to 24 h (8). Since AmB was only static at clinically achievable concentrations (Fig. 2), the longer the incubation time, the greater the regrowth of the organism as the total AmB loses its activity. Gold et al. (9) found that the tolerance of their *A. fumigatus* isolates to AmB increased markedly when the time of incubation was increased from 24 to 48 h. On this basis, we think that the shortest possible time of incubation for susceptibility tests is the most valid. The susceptibility tests in this report were read after 24 h of incubation. When we correct for the decay of AmB activity after 24 h, the actual MIC values may actually be half of what we obtained.

We have been evaluating a radioactive susceptibility test based on the incorporation of radioactive precursors into RNA (3). Using this method, we have been able to determine the susceptibility of slow-growing organisms to AmB much more rapidly than with the traditional methods. Our initial evaluation of this method with *Aspergillus* showed that [^{3}H]uridine was incorporated into RNA, and that the incorporation could be followed by determining the incorporation of radioactivity into acid-precipitable fractions of whole organisms. In addition, the AmB dose response curve for [^{3}H]uridine incorporation into *Aspergillus* RNA was similar to our other studies in that the 50% inhibition of RNA synthesis (13) correlated best with the MIC obtained.

For 5FC and rifampin, however, the visual-turbidity MIC was much higher than the 50% inhibition of RNA synthesis and dry weight. We are not sure of the reason for this discrepancy. It is probable that turbidity measured visually is not as sensitive as the more quantitative and specific effects of drug action. In the case of 5FC and rifampin, these agents specifi-

cally inhibit RNA synthesis (2, 13), hence measurements of RNA synthesis should be the most sensitive assay of their effects. On the other hand, AmB affects membrane permeability (11), and measurements specific for this effect would be more sensitive than measurements of turbidity or RNA synthesis. Additional experiments done in our laboratory have confirmed this latter hypothesis (J. K. Brajtburg et al., unpublished data).

It is extremely important to determine which of the indexes we measured is the most clinically relevant in drug susceptibility tests with *Aspergillus*. If turbidity in Salvin medium measures true susceptibility, then rifampin and 5FC will not be clinically useful against *Aspergillus* infections because the MICs we obtained by using this method were above clinically achievable blood levels. If RNA synthesis or dry weight in Salvin medium is the most relevant measure of in vivo susceptibility, these drugs may be clinically useful against *Aspergillus* infection. Although the clinical reports describing successful treatment of *Aspergillus* infections with 5FC suggest that RNA and dry-weight determinants may be valid indications of drug effectiveness (1), animal experiments and further experience with human infections are needed to confirm this. Finally, it is clear from our studies that susceptibility testing in *Aspergillus* gives variable results depending on the conditions used. We believe that until a standardized technique that can be shown to reflect in vivo results is developed, decisions regarding the choice of antifungal agents for therapy of clinical infections should not be based solely on in vitro susceptibility testing.

ACKNOWLEDGMENTS

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