

Variables Influencing Susceptibility Testing of *Cryptococcus neoformans* to 5-Fluorocytosine

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The minimum inhibitory concentration (MIC) of 5-fluorocytosine (5-FC) was determined for 65 isolates of *Cryptococcus neoformans* by using a twofold serial tube dilution method. The MIC was profoundly influenced by incubation temperature, inoculum size, and duration of incubation. By using a standard set of test conditions, 100% of 49 pretreatment isolates of *C. neoformans* were susceptible to 10 μg of 5-FC per ml or less, and 9 (56%) of 16 isolates recovered during or after 5-FC therapy were massively drug resistant (MIC > 320 $\mu\text{g}/\text{ml}$). With the standard test conditions recommended here, the tube dilution method was found to be both accurate and reproducible, and the results correlated with the treatment status of patients.

5-Fluorocytosine (5-FC) is an oral antifungal agent synthesized in 1957 and shown in 1963 to be effective in the treatment of mice infected experimentally with *Cryptococcus neoformans* (2). Since 1967, 5-FC has been used with limited success in the therapy of cryptococcal infections in man (9, 11). Clinical use of 5-FC must be predicated upon adequate identification of the infecting organism and accurate, reproducible in vitro measurements of drug susceptibility.

To date, nearly 100% of pretreatment isolates of *C. neoformans* studied have been found to be sensitive in vitro to less than 10 μg of 5-FC per ml (5, 6, 8). These results have been generated in one laboratory by using a tube dilution method with a single set of test conditions. More recently, several groups have noted that changes in inoculum size or time of incubation may produce marked differences in susceptibility results with a variety of pathogenic *Candida* (4, 7, 10). To ascertain whether in vitro testing of *C. neoformans* is similarly affected, to delineate the more important variables influencing 5-FC susceptibility testing of *C. neoformans*, and to help establish guidelines ensuring reproducible in vitro test results, we investigated the effects of inoculum size, incubation temperature, and time of incubation on the minimal inhibitory concentration (MIC) of 65 isolates of *C. neoformans*.

MATERIALS AND METHODS

Clinical isolates. Sixty-five strains of *C. neoformans* isolated from 50 patients were studied. These

included 32 strains from 22 patients with cryptococcosis treated with 5-FC at the National Institutes of Health (NIH) since 1968 and 33 isolates from 28 patients referred for testing. Each organism to be tested was subcultured on yeast-nitrogen base (YNB; Difco) agar slants incubated at 30 C for 48 h, and then was harvested with sterile sodium phosphate-buffered normal saline (0.15 M saline, 0.0008 M phosphate buffer, pH 7.0; PBS). These saline suspensions were adjusted to an optical density of 0.300 (saline blank) at 600 nm ($3.0 \pm 0.5 \times 10^7$ viable cells/ml) in a Coleman Junior spectrophotometer with a 10 \times 75 mm round cuvette.

Preparation of media and 5-FC. YNB was diluted 1:10 with sterile, distilled water, and each 100 ml was supplemented with 6 ml of 50% glucose. Solutions of 5-FC were prepared in PBS and sterilized by filtration. Twofold serial dilutions of drug, from 2.5 to 320 $\mu\text{g}/\text{ml}$, were used as final concentrations in all studies.

Reading results. 5-FC MIC was determined by a broth dilution method in which all tubes, containing a final volume of 5 ml, were rotated on a drum at 2 rpm throughout the period of incubation. In the absence of skipped tubes, the MIC was defined as the lowest concentration of drug in which no visible growth (i.e., turbidity) is observed. Skipped tubes refer to the appearance of growth in one or more 5-FC-containing tubes without concomitant turbidity in all tubes containing lower concentrations of drug.

RESULTS

Effect of inoculum size and temperature. 5-FC MIC were determined after 48 h of incubation at 32 C for 20 isolates of *C. neoformans* by using two different inocula (Fig. 1). Eighteen (90%) isolates were inhibited by 10 μg

of 5-FC per ml or less at the lower inoculum, whereas only 65% were inhibited at the same drug concentration at the higher inoculum size. Similar differences were observed when 48-h MIC were determined on 11 strains of *C. neoformans* incubated at either 32 or 37 C by using an inoculum of 6×10^4 organisms/tube (Fig. 2). Nine isolates (82%) had identical MIC at both temperatures studied, whereas two isolates (18%) were 5-FC resistant (MIC > 320 $\mu\text{g/ml}$) at 32 C, but exquisitely sensitive (MIC ≤ 2.5 $\mu\text{g/ml}$) at 37 C.

Effect of incubation time. Results of 5-FC MIC determined daily for 5 days in *C. neoformans* incubated at 32 C are shown in Tables 1 and 2. Fifty-five isolates were studied by using a low inoculum (Table 1), and in no instance was growth seen at 24 h. At 48 h of incubation, all 55 isolates were sensitive to 10 μg of 5-FC per ml or less, and no skipped tubes were observed. Thirty-three (60%) of these 55 isolates had no change in MIC during the last 3 days of incubation. Five (9%) of the 55 isolates became resist-

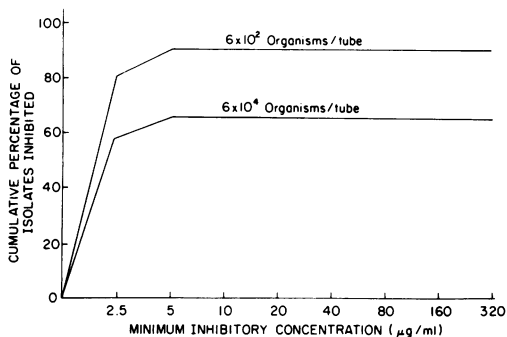


FIG. 1. 5-FC minimal inhibitory concentrations for 20 isolates of *C. neoformans* studied at two different inoculum sizes. Results were read after 48 h of incubation at 32 C.

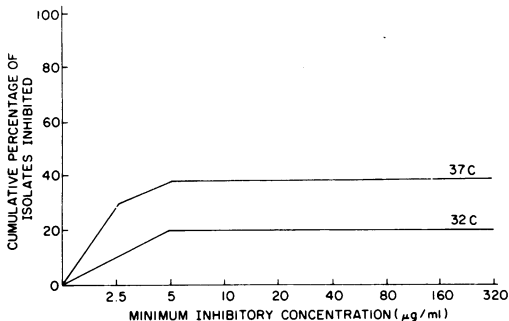


FIG. 2. 5-FC minimal inhibitory concentrations (48-h reading) for 11 isolates of *C. neoformans* studied at two different temperatures. Inoculum was 6×10^4 organisms/tube.

TABLE 1. Cumulative percentage of 55 isolates of *Cryptococcus neoformans* found to be susceptible (MIC ≤ 10 $\mu\text{g/ml}$) or resistant (MIC > 320 $\mu\text{g/ml}$) to 5-FC or to have developed skipped tubes during daily *in vitro* susceptibility testing^a

Isolate susceptibility	Incubation time (days)				
	1	2	3	4	5
Sensitive	No growth	100	82	75	60
Resistant	No growth	0	4	4	9
Skipped tube(s)	No growth	0	14	21	31

^a Testing was for 5 days at 32 C with an inoculum of 6×10^2 organisms/tube.

TABLE 2. Cumulative percentage of 15 isolates of *Cryptococcus neoformans* found to be susceptible (MIC ≤ 10 $\mu\text{g/ml}$) or resistant (MIC > 320 $\mu\text{g/ml}$) to 5-FC or to have developed skipped tubes during daily *in vitro* susceptibility testing^a

Isolate susceptibility	Incubation time (days)				
	1	2	3	4	5
Sensitive	100	75	20	13	7
Resistant	0	25	40	40	40
Skipped tube(s)	0	0	40	47	53

^a Testing was for 5 days at 32 C with an inoculum of 6×10^4 organisms/tube.

ant to this drug with incubation beyond 48 h. In addition, growth occurred in one or more skipped tubes in the remaining 17 (31%) isolates between day 3 and day 5, interfering with subsequent interpretation of the MIC. Subcultures of the organisms from these skipped tubes were found to be massively resistant to 5-FC (MIC > 1,000 $\mu\text{g/ml}$) in the six instances in which it was checked.

Nine of the 33 organisms in Table 1 which exhibited no change in MIC between day 2 and day 5 were pretherapy isolates from nine patients, respectively, with cryptococcosis who were cured with 5-FC at the NIH. Six similarly treated patients failed to respond to 5-FC because of the development of drug-resistant organisms during or after therapy. Pretherapy isolates from these six patients were sensitive to 10 μg of 5-FC per ml after 48 h of incubation at 32 C, but all six developed one or more skipped tubes on the 3rd day of incubation. Thus, the appearance of skipped tubes after 72 h (or more) of incubation under the conditions described here in a pretreatment isolate of *C. neoformans* may be useful in predicting subsequent development of *in vivo* drug resistance. No clear clinical correlation can be made between the

response to 5-FC and MIC results for the other 40 isolates (35 patients) reported in Table 1.

Results of the tube dilution sensitivities from 15 isolates of *C. neoformans*, by using a high initial inoculum (6×10^4 organisms/tube), are shown in Table 2. All 15 isolates were sensitive to 10 μg of 5-FC per ml or less after 24 h of incubation, but only one (7%) isolate had no change in sensitivity during the last 4 days of incubation. Fifty-three percent of isolates developed skipped tubes, and another 40% became massively resistant to 5-FC between day 2 and day 5 of incubation.

Standardized sensitivity testing. When it was realized that temperature, incubation time, and inoculum size had such profound effects upon 5-FC MIC for *C. neoformans*, we repeated the tube dilution sensitivities on our 65 isolates of cryptococci by using a set of standard test conditions. In all instances, MIC were read after 48 h at 32 C with an initial inoculum of 6×10^2 organisms/tube (Table 3). All pretreatment isolates were sensitive to 10 μg of 5-FC per ml or less, whereas 9 (56%) of 16 isolates recovered during or after 5-FC therapy were massively resistant. Skipped tubes did not appear in any isolate under these test conditions, and MIC values were quite reproducible upon retesting.

DISCUSSION

Our results demonstrate for the first time that even slight changes in inoculum size and temperature and time of incubation can significantly influence the resultant 5-FC MIC in *C. neoformans*. In general, large inocula and long incubation periods at lower temperatures (30 to 32 C) will increase the number of 5-FC resistant cryptococci reported from a given laboratory. Smaller inocula and shorter incubation times at 37 C will favor lower MIC values. Thus it seems clear that interlaboratory comparison of MIC values for *C. neoformans* is meaningful only if test conditions are comparable.

The marked temperature effect which we noted in 2 of 11 isolates of *C. neoformans* may

be related to a slower growth rate of cryptococci at 37 C. With a longer growth period (i.e., incubation time), these two isolates may have exhibited resistance to 5-FC at 37 C as well. The mechanism for the shifts in 5-FC inhibitory end points and the development of skipped tubes with increasing incubation time is not known, but similar shifts have been reported with *Candida albicans* (4). Drug inactivation or degradation has been excluded by unpublished studies from our laboratory which indicate that 5-FC is stable with no loss of in vitro bioactivity when incubated with or without live organisms at 30, 37, or 56 C for 120 h.

When MIC were determined after 48 h of incubation on a rotating drum (2 rpm) at 32 C with an inoculum of 6×10^2 organisms/tube, a bimodal distribution of MIC values was observed (Table 3). This distribution has been noted previously and is due to the uniform sensitivity of pretreatment strains of *C. neoformans* and the selection of drug-resistant isolates during therapy (1, 8). Seventeen of the 65 strains reported here were isolates from our own patients which were included in a previous report by Shadomy (6). MIC values differed by more than two tubes in 5 (30%) of these 17 isolates, and these results were most likely due to interlaboratory differences in incubation temperature and inoculum size. When temperature, inoculum size, and incubation time were standardized as recommended here, the 48-h MIC results had sharply defined end points which were reproducible, free of skipped-tube phenomena, and correlated with the treatment status observed in patients studied at the NIH. This recommended method is easily mastered, but suffers from being both time consuming and costly. To date, in vitro 5-FC disk susceptibility testing of *C. neoformans* has not been reported, but investigation is warranted in view of encouraging preliminary results with other pathogenic fungi (3).

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TABLE 3. 5-FC minimal inhibitory concentrations after 48 h of incubation at 32 C for 65 strains of *C. neoformans* isolated from 50 patients^a

Treatment and total no. of isolates/patients	No. inhibited at various concentrations ($\mu\text{g}/\text{ml}$) of 5-FC							
	≤ 2.5	5.0	10	20	40	80	160	> 320
Pretreatment, 49/38	47/36	2/2	0	0	0	0	0	0
During or after 5-FC therapy, 16/12	6/3	0	0	0	1/1	0	0	9/8

^a Inoculum size was 6×10^2 organisms/tube.

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