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# In Vitro Activity of 5-Fluorocytosine Against *Candida* and *Torulopsis* Species

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One hundred yeasts were studied. Tests included detailed identification and determination of 24- and 48-hr minimal inhibitory concentrations and minimal fungicidal concentrations of 5-fluorocytosine (5-FC). Final identifications included 57 isolates of *Candida albicans*, 15 isolates of *C. tropicalis*, 13 isolates of *C. parapsilosis*, 7 isolates of *Torulopsis glabrata*, 3 isolates of *C. guilliermondii*, 2 isolates each of *C. stellatoidea* and *Cryptococcus neoformans*, and 1 isolate of *Candida krusei*. Twenty-three original identifications were in error; these involved mostly *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *T. glabrata*. Inhibitory end point readings based on 24 hr of incubation were misleading. Whereas 79 of 91 isolates of *Candida* appeared to be inhibited at 24 hr by 12.5 μg or less of 5-FC/ml, only 52 were inhibited at 48 hr; whereas only 12 isolates appeared to be resistant to 100 μg/ml after 24 hr, 37 were resistant after 48 hr. Susceptibility varied among the different species. *C. tropicalis* was the most susceptible, with 10 of 15 isolates (66.7%) being inhibited by 12.5 μg or less/ml and 7 (46.7%) being killed by 100 μg or less/ml. *C. albicans* was similarily susceptible; 33 of 57 isolates (57.9%) were inhibited by 12.5 μg or less/ml and 25 (43.9%) were killed by 100 μg or less/ml. *C. parapsilosis* was quite resistant, as only 4 of 13 isolates (30.8%) were inhibited by 12.5 μg or less/ml and 3 (23.1%) were killed by 100 μg or less/ml.

In a previous report (6) it was noted that 8 of 15 clinical isolates of *Candida* species (53%) were resistant to 100 μg or less of 5-fluorocytosine (5-FC) per ml. It also was stated that susceptibility did not appear to be related to speciation. However, subsequent experience has indicated that, whereas the proportion of susceptible strains of *Candida* species has remained unchanged, the matter of speciation may be critical in predicting susceptibility and making decisions regarding clinical use of this drug.

This study presents susceptibility data for 91 isolates of *Candida* species and 7 isolates of *Torulopsis glabrata*, and also examines the relationship between species and susceptibility.

## MATERIALS AND METHODS

**Cultures.** One hundred yeasts were studied. These included 75 previously studied isolates from our stock culture collection, 21 recovered more recently from clinical material either at this institution or elsewhere and 4 cultures received through the Proficiency Testing Program in Mycology administered by the Center for Disease Control, Atlanta, Ga. All cultures were maintained on drug-free modified Sabouraud agar with 20% dextrose (Difco).

**Identification procedures.** All yeasts were identified on the basis of sugar fermentation and assimilation reactions, microscopic morphology, production of chlamydospores and germ tubes and, when required, urease activity and nitrate assimilation.

Fermentation tests employed screw-cap tubes (12 by 150 mm) containing 2 ml of a base medium consisting of yeast extract (0.45%), peptone (0.75%), and bromothymol blue, sterilized together with Durham tubes. Volumes of 1 ml of filter-sterilized solutions (6%) of appropriate sugars were added to the tubes prior to inoculation. These included dextrose, maltose, sucrose, lactose, galactose, and trehalose. Sugar assimilation was determined in 5-ml volumes of yeast nitrogen base (Difco) to which specific carbon sources were added. These included cellobiose, xylose, trehalose, and inositol, which were used at a final concentration of 5%, and raffinose, which was used at a final concentration of 10%. Inocula were adjusted to an approximate light transmission of 70%; 0.05 to 0.10-ml volumes were added to each fermentation or assimilation tube.

Nitrate assimilation tests were performed by the auxanographic method. A 2-ml amount of sterile 10× yeast carbon base (Difco) was mixed with 10 ml of sterile, melted 2% Noble's agar, and the mixture was poured into a petri dish previously seeded with 0.1 ml of a yeast suspension. The contents of the plate were mixed and allowed to harden, after which several crystals of $KNO_3$ were dropped on one side and an equal amount of peptone was placed on the other. Urease activity was determined on Christensen's urea agar (Difco).

Production of chlamydospores was determined on commercially prepared cornmeal agar (Difco) by use of the Dalmau inoculation technique. Production of germ tubes was determined by preparing light suspensions of yeast cells in 1.0-ml volumes of sterile, normal human serum with incubation at 37 C for 3 hr before microscopic examination.

Except where noted, all tests were incubated at room temperature. Urease and nitrate assimilation tests were read at 48 hr; fermentation and assimilation tests were read periodically, with final readings after 2 weeks.

**Susceptibility tests.** Susceptibility to 5-FC was measured in yeast nitrogen base (Difco) supplemented with dextrose and L-asparagine. Incubation was at 30 C. Concentrations of drug ranged from 100 to 0.05 μg/ml. Minimal inhibitory concentrations were read after 24 and 48 hr of incubation, and plates of Sabouraud agar were inoculated after 24 hr to determine minimal fungicidal concentrations. *Saccharomyces cerevisiae* ATCC 9763 was included as a control in each set of determinations. Further details regarding susceptibility testing have been published elsewhere (5).

## Results

The most striking result, and by far the most disturbing, was the large number of misidentified cultures in our stock culture collection. Of 75 cultures studied, 64 represented clinical isolates or Proficiency Testing Program unknowns, and the remaining 11 were known or reference organisms. Of the 64 unknown organisms, 22 (34.4%) apparently had been misidentified either in our laboratory or elsewhere on initial isolation. In addition, two cultures of *Cryptococcus neoformans* had been catalogued incorrectly as *Candida albicans*. The results of the reidentifications are shown in Table 1. It should be noted that these studies were done without knowledge of prior identifications.

Final identifications of 25 more recent isolates were as follows: *C. albicans*, 20; *C. tropicalis*, 2, including one initially identified elsewhere as *C. parapsilosis; C. parapsilosis*, 1; *C. guilliermondii*, 1; and *T. glabrata*, 1.

The susceptibility of the 91 isolates of *Candida* to 5-FC was determined. Analysis of these data revealed two important findings.

First, 24-hr readings resulted in erroneously low minimal inhibitory concentrations (Table 2); of 79 isolates which appeared to be inhibited by 12.5 μg or less/ml at 24 hr, only 52 (65.8%) were inhibited by similar concentrations after 48 hr. More importantly, whereas only 12 isolates grew out in the presence of 100 μg/ml after 24 hr, 37 grew in this concentration after 48 hr. Concentrations of 100 μg or less/ml were fungicidal for 39 isolates, and 52 isolates were totally resistant to 5-FC (Table 2).

Second, susceptibility varied according to species (Tables 3–5). Based on 48-hr readings, 33 of 57 isolates of *C. albicans* (57.9%) were inhibited by 12.5 μg/ml, and 22 (38.6%) were killed; 24 isolates (42.1%) were totally resistant. In contrast, 4 of 13 isolates of *C. parapsilosis* (30.8%) were inhibited by similar concentrations, and 3 (23.1%) were killed. *C. tropicalis* was the most susceptible, with 10 of 15 isolates (66.7%) being inhibited by 6.25 μg or less/ml and 7 (46.7%) being killed by 50 μg or less/ml.

Six isolates of three other species of *Candida* also were tested. Two isolates of *C. stellatoidea* and one isolate of *C. guilliermondii* were killed by 0.78 μg/ml. Two additional isolates of *C. guilliermondii* were inhibited by 3.13 μg/ml, but one was not killed by less than 50 μg/ml and the second by not less than 100 μg/ml. One isolate of *C. krusei* was inhibited at 25 μg/ml but not killed by 100 μg/ml.

All seven isolates of *T. glabrata* were susceptible to 5-FC. Six were inhibited by 0.78 μg or less/ml and the seventh by 50 μg/ml ($\bar{X}$ = 7.4 μg/ml; $s$ = 18.8). Four were killed by 0.20 μg/ml and a fifth by 50 μg/ml ($\bar{X}$ = 10.1 μg/ml; $s$ = 22.3); two were not killed by 100 μg/ml.

## DISCUSSION

5-FC offers an attractive alternative to amphotericin B and nystatin in the treatment of serious life-threatening or systemic infections caused by yeast-like organisms. However, its clinical use must be predicated on adequate knowledge of the infecting organism. Data presented here identify two particular items of information which must be available to the clinician who is using 5-FC in the treatment of infections caused by members of the genus *Candida*. These are, first, an accurate in vitro measurement of susceptibility and, second, reliable identification of the infecting organism.

The question of selection of media for susceptibility testing with 5-FC and *Candida* or *Cryptococcus* isolates has already been examined (5, 6). One question which was examined

TABLE 1. *Results of reidentification studies of 75 previously studied yeasts*

| Final identifications[a] | Total no. | No. of unknowns[b] | Percent of error | Prior identifications |
|---|---|---|---|---|
| *Candida albicans* | 37 | 30 | 16.7 | *C. albicans*, 32<br>*C. parapsilosis*, 2<br>*C. krusei*, 1<br>*C. stellatoidea*, 1<br>*C. tropicalis*, 1 |
| *C. tropicalis* | 13 | 11 | 72.7 | *C. albicans*, 7<br>*C. tropicalis*, 5<br>*C. parapsilosis*, 1 |
| *C. parapsilosis* | 12 | 12 | 41.7 | *C. parapsilosis*, 7<br>*C. albicans*, 4<br>*C. tropicalis*, 1 |
| *Torulopsis glabrata* | 6 | 5 | 60.0 | *T. glabrata*, 3<br>*C. parapsilosis*, 2<br>*C. tropicalis*, 1 |
| *C. guilliermondii* | 2 | 2 | 50 | *C. guilliermondii*, 1<br>*C. tropicalis*, 1 |
| *C. stellatoidea* | 2 | 2 | 0 | *C. stellatoidea*, 2 |
| *C. krusei* | 1 | 0 | 0 | *C. krusei*, 1 |
| *Cryptococcus neoformans* | 2 | 1 | 100 | *C. albicans*, 2 |

[a] Final identifications based on sugar fermentation and assimilation reactions.

[b] "Unknowns" include both clinical isolates and cultures received through the Proficiency Testing Program in Mycology administered by the Center for Disease Control, Atlanta, Ga.

here was that of time of incubation. Our data suggest that 24 hr is not adequate for detection of 5-FC resistance in *Candida* isolates. Previous unpublished studies have indicated that 5-FC is a relatively stable compound with no significant loss of in vitro activity when incubated at either 30 or 37 C for over 96 hr. Thus, differences between inhibitory end points as measured at 24 and 48 hr must be attributed to the metabolic responses of tested organisms.

Schönebeck (4) has presented a possible explanation for shifts in inhibitory end points of 5-FC after continued incubation of *Candida* species. He describes two physiological classes of 5-FC-resistant mutants. Class 1 is totally resistant to 5-FC as well as to 5-fluorouracil and 5-fluorouridine. Schönebeck suggested that the mutational site in this class may involve gene coding for uridine monophosphate pyrophosphorylase. The second class, and the most frequent among clinical isolates, is inhibited through 72 hr of incubation but grows in the presence of high concentrations of 5-FC after 3 to 7 days. In these organisms, decreased susceptibility to 5-FC is associated with reduced incorporation of free exogenous $^{3}$H-uridine into ribonucleic acid. Schönebeck interpreted this finding, coupled with resistance to 5-fluorouridine, as showing that neither cytosine permeases nor cytosine deaminases are involved. Infections due to isolates of *Candida* with this second class of resistance, in Schönebeck's opinion, may still be treatable with 5-FC.

The second item of information which was examined in this study was the relationship between susceptibility to 5-FC and species identification. Although the data presented here demonstrate a specific relationship between *Candida* speciation and susceptibility to 5-FC, they also demonstrate a significant problem relative to methods of identification. As previously noted, 23 of the 98 isolates of *Candida* and *Torulopsis* species, or 23.5%, included in this study had been misidentified. Sixteen were misidentified in our laboratory; the remaining seven were received and catalogued as identified without confirmation. Examination of prior records and a review of the various methods for yeast identification employed in our laboratory shed some light on this problem.

Two different procedures for yeast identification have been used over the past 6 years. The first was based on that of Ajello et al. (2). The second, adopted in late 1970, was based on that of Haley (3). There are several major differences between the two procedures for determination of sugar fermentation and assimilation reactions. The method described by Ajello et al. employs four sugars in the fermentation tests (dextrose, maltose, sucrose, and lactose prepared in a beef extract base) and an auxanographic assimilation method. That of Haley employs five sugars in the fermentation

TABLE 2. *In vitro susceptibility of 91 isolates of Candida species to 5-fluorocytosine after 24 and 48 hr of incubation*

| Concn (μg/ml) | Minimal inhibitory concn | | Minimal fungicidal concn |
|---|---|---|---|
| | 24 hr | 48 hr | |
| >100 | 12 (100)[a] | 37 (100) | 52 (100) |
| 100 | — | — | 2 (42.9) |
| 50 | — | 1 (59.3) | 3 (40.7) |
| 25 | — | 1 (58.2) | 2 (37.4) |
| 12.5 | 2 (86.8) | 2 (57.1) | — |
| 6.25 | 1 (84.6) | 2 (55.0) | 1 (35.2) |
| 3.13 | 4 (83.5) | 3 (52.8) | 1 (34.1) |
| 1.56 | 7 (79.1) | 4 (49.5) | 3 (33.0) |
| 0.78 | 7 (71.4) | 10 (45.1) | 8 (29.7) |
| 0.39 | 10 (63.7) | 13 (34.1) | 11 (20.9) |
| 0.20 | 29 (52.8) | 14 (19.8) | 6 (8.8) |
| 0.10 | 14 (20.9) | 3 (4.4) | 2 (2.2) |
| ≤0.05 | 5 (5.5) | 1 (1.1) | — |
| Number susceptible | 79 | 54 | 39 |
| $\bar{X}$ | 0.91 | 2.67 | 10.93 |
| $s$ | 2.12 | 7.74 | 25.21 |

[a] Number of strains for which the indicated 5-fluorocytosine concentration was the minimal inhibitory or minimal fungicidal concentration, with the accumulative percentage of strains given in parentheses.

tests (the above, plus galactose, prepared in a yeast extract and peptone base) and the Wickerham tube assimilation test.

Isolates of *Candida* most frequently misidentified included *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. All three belong in Fell and Meyer's group V in terms of oxidative assimilation reactions (*unpublished data*, cited by Ahearn [1]). This group is characterized by being positive for trehalose but negative for nitrate, inositol, lactose, raffinose, and cellobiose. The principal problems encountered in identifying these organisms by the method of Ajello et al. (2) included variations in sucrose fermentation reactions and in galactose and cellobiose assimilations. For example, of 23 isolates of *C. albicans* which were originally identified by this method, 4 were misidentified as *C. parapsilosis* (2 isolates) and 1 each as *C. stellatoidea* or *C. tropicalis*. The primary source of error in all four was variation in sucrose fermentation; additional sources included misreading of auxanographic assimilations of galactose and variations in cellobiose assimilation. Similarly, 4 of 13 isolates of *C. parapsilosis* and 8 of 15 isolates of *C. tropicalis* were originally misidentified, mostly as *C. albicans*. In most instances, the primary source of error was again variability in sucrose fermentation and auxanographic cellobiose assimilation.

The inclusion of galactose in the fermentation pattern and use of the Wickerham tube assimilation test eliminated most of the problems caused by variations in fermentation and assimilation reactions. Although no pattern is absolute, the following patterns based on reactions obtained with maltose, sucrose, and galactose helped identify the group V organisms: fermentation of maltose and galactose, and assimilation of sucrose—*C. albicans;* fermentation of maltose, sucrose, and galactose—*C. tropicalis;* assimilation of maltose and galactose, and fermentation of sucrose—*C. parapsilosis*.

Another diagnostic problem was *T. glabrata*. Four of seven isolates were correctly identified by use of the method of Haley (3) plus inclusion of an additional trehalose fermentation tube. Two isolates originally identified according to the method of Ajello et al. (2) were misidentified as *C. parapsilosis;* one was received identified as *C. tropicalis*.

It should be noted that 22 of 23 isolates for which there was disagreement between initial and subsequent identification were submitted to the Mycology Training Unit, Center for Disease Control, for identification. With 19, there

TABLE 3. *In vitro susceptibility of 57 isolates of Candida albicans to 5-fluorocytosine after 24 and 48 hr of incubation*

| Concn (μg/ml) | Minimal inhibitory concn | | Minimal fungicidal concn |
|---|---|---|---|
| | 24 hr | 48 hr | |
| >100 | 8 (100)[a] | 24 (100) | 32 (100) |
| 100 | — | — | 2 (43.9) |
| 50 | — | — | — |
| 25 | — | — | 1 (40.4) |
| 12.5 | 1 (86.0) | 2 (57.9) | — |
| 6.25 | 1 (84.2) | 1 (54.4) | 1 (38.6) |
| 3.13 | 3 (82.5) | 1 (52.6) | 1 (36.8) |
| 1.56 | 6 (77.1) | 2 (50.9) | 1 (35.1) |
| 0.78 | 3 (66.7) | 5 (47.4) | 5 (33.3) |
| 0.39 | 4 (61.4) | 7 (38.6) | 8 (24.6) |
| 0.20 | 22 (54.4) | 12 (26.3) | 4 (10.5) |
| 0.10 | 7 (15.8) | 2 (5.3) | 2 (3.5) |
| ≤0.05 | 2 (3.5) | 1 (1.8) | — |
| Number susceptible | 49 | 33 | 25 |
| $\bar{X}$ | 0.95 | 1.42 | 9.76 |
| $s$ | 2.03 | 3.08 | 27.61 |

[a] Number of strains for which the indicated 5-fluorocytosine concentration was the minimal inhibitory or minimal fungicidal concentration, with the accumlative percentage of strains given in parentheses.

TABLE 4. *In vitro susceptibility of 13 isolates of Candida parapsilosis to 5-fluorocytosine after 24 and 48 hr of incubation*

| Concn (μg/ml) | Minimal inhibitory concn | | Minimal fungicidal concn |
|---|---|---|---|
| | 24 hr | 48 hr | |
| >100 | 3 (100)[a] | 9 (100) | 10 (100) |
| 100 | — | — | — |
| 50 | — | — | — |
| 25 | — | — | — |
| 12.5 | — | — | — |
| 6.25 | — | — | — |
| 3.13 | 1 (76.9) | 1 (30.8) | — |
| 1.56 | — | — | 1 (23.1) |
| 0.78 | 2 (69.2) | — | — |
| 0.39 | 1 (53.9) | 2 (23.1) | 2 (15.4) |
| 0.20 | 1 (46.2) | 1 (7.7) | — |
| 0.10 | 4 (38.5) | — | — |
| ≤0.05 | 1 (7.7) | — | — |
| Number susceptible | 10 | 4 | 3 |
| $\bar{X}$ | 0.57 | 1.03 | 0.78 |
| $s$ | 0.94 | 1.40 | 0.68 |

[a] Number of strains for which the indicated 5-fluorocytosine concentration was the minimal inhibitory or minimal fungicidal concentration, with the accumulative percentage of strains given in parentheses.

was full agreement as to final identification. Two identifications of *C. albicans* by the Center for Disease Control were rejected because both their and our fermentation and assimilation patterns were more compatible with *C. tropicalis*. A third was rejected because both their results and ours were more compatible with *C. parapsilosis*.

The data presented here regarding in vitro susceptibility of *Candida* isolates to 5-FC are in partial disagreement with those recently reported by Steer et al. (7). They reported that 23 of 25 isolates, or 92%, of *C. albicans*, and 9 of 11 isolates, or 82%, of other species were inhibited by 1.95 μg or less/ml as determined by microtiter dilution after 48 hr of incubation at 37 C. In contrast, we found 50.9% of *C. albicans* isolates, 60.0% of *C. tropicalis* isolates, and 30.8% of *C. parapsilosis* isolates to be inhibited by 1.56 μg/ml, the nearest corresponding dilution in our study. However, corresponding data for fungicidal levels are in closer agreement. They found that 76% of their strains of *C. albicans* and 90% of other *Candida* species required at least 15 μg/ml for killing. Our data, with 12.5 μg/ml as the nearest corresponding fungicidal level, were as follows: all 91 isolates, 57.2%; *C. albicans*, 56.1%; *C. parapsilosis*, 76.9%; *C. tropicalis*, 53.3%. The most likely explanation for these differences can be found in the methods employed. They used a microtiter method, whereas we used a broth dilution procedure. In our method, any trace amount of growth after 48 hr of incubation greater than that seen in a distilled water control was regarded as positive. It is likely that such traces would have been missed in the microtiter procedure. Another factor which must be considered is the nature of inoculum. In our studies, the inocula contained approximately $10^5$ cells harvested from Sabouraud agar plates and were delivered into 1.0-ml test volumes. In the study of Steer et al. (7), inocula were prepared by dilution of overnight Sabouraud broth cultures and probably contained no more than $10^4$ cells in a test volume of 0.05 ml. It is possible that these differences in both density and sources of inocula may account for the differences in susceptibility test results. Recent results (Shadomy and Davis, *unpublished data*) with *Candida* do, indeed, suggest that inoculum size may be important in susceptibility testing with 5-FC. This problem is presently under examination.

There is closer agreement between our results and those of Steers et al. (7) in data for *T. glabrata*. They reported that 39 of 47 isolates, or 83%, were inhibited and 38, or 81%, were

TABLE 5. *In vitro susceptibility of 15 isolates of Candida tropicalis to 5-fluorocytosine after 24 and 48 hr of incubation*

| Concn (μg/ml) | Minimal inhibitory concn | | Minimal fungicidal concn |
|---|---|---|---|
| | 24 hr | 48 hr | |
| >100 | 1 (100)[a] | 4 (100) | 8 (100) |
| 100 | — | — | — |
| 50 | — | 1 (73.3) | 2 (46.7) |
| 25 | — | — | 1 (33.3) |
| 12.5 | — | — | — |
| 6.25 | — | 1 (66.7) | — |
| 3.13 | — | — | — |
| 1.56 | 1 (93.3) | 1 (60.0) | 1 (26.7) |
| 0.78 | 2 (86.7) | 4 (53.3) | 1 (20.0) |
| 0.39 | 4 (73.3) | 2 (26.7) | — |
| 0.20 | 5 (46.7) | 1 (13.3) | 2 (13.3) |
| 0.10 | — | 1 (6.6) | — |
| ≤0.05 | 2 (13.3) | — | — |
| Number susceptible | 14 | 11 | 7 |
| $\bar{X}$ | 0.41 | 5.64 | 18.25 |
| $s$ | 0.40 | 14.82 | 23.44 |

[a] Number of strains for which the indicated 5-fluorocytosine concentration was the minimal inhibitory or minimal fungicidal concentration, with the accumulative percentage of strains given in parentheses.

killed by 1.95 μg or less/ml. In our study, 85.7 and 57.1% were inhibited and killed, respectively, by 1.56 μg/ml. Neither interpretation of trace residues nor shifts in inhibitory end points between 24- and 48-hr readings were as serious a problem with *T. glabrata* as they were with *Candida* species.

In the final analysis, three points are evident. First, sound clinical use of 5-FC requires optimal laboratory support, particularly as it applies to susceptibility testing and yeast identification. Second, although broth dilution procedures are tedious and expensive, they may be more reliable than other procedures for susceptibility testing, particularly because they can also give an indication of fungicidal activity of 5-FC. Third, regardless of how hard the laboratory may be pressed to provide 5-FC susceptibility end points, a 24-hr reading may be misleading and should be regarded as potentially more harmful than no data at all.

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