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# Fluconazole Susceptibility Testing of *Cryptococcus neoformans*: Comparison of Two Broth Microdilution Methods and Clinical Correlates among Isolates from Ugandan AIDS Patients

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**We compared the yeast nitrogen base (YNB) broth microdilution method with the National Committee for Clinical Laboratory Standards (NCCLS) M27-A microdilution reference method for measuring the in vitro susceptibility of *Cryptococcus neoformans* isolates to fluconazole. A total of 149 isolates of *C. neoformans* var. *neoformans* from Ugandan AIDS patients was tested by both methods. An overall agreement of 88% between the two microdilution methods was observed. All isolates grew well in both RPMI 1640 and YNB media, and MICs could be read after 48 h of incubation by both methods. The range of fluconazole MICs obtained with the YNB method was broader than that obtained with the NCCLS method. The extended range of MICs provided by the YNB method may be of clinical value, as it appears that the clinical outcome may be better among patients infected with strains inhibited by lower concentrations of fluconazole as determined by the YNB method. The YNB method appears to be a viable option for testing *C. neoformans* against fluconazole.**

*Cryptococcus neoformans* causes serious and even life-threatening central nervous system infections in immunocompromised individuals (3, 4, 12, 22) and remains incurable in patients with AIDS (1, 3, 4, 12, 15, 22). Azoles are used extensively for prophylaxis, treatment, and long-term maintenance of serious fungal infections in the AIDS population (2, 3, 12, 15). Such long-term exposure may lead to the development of resistant strains (1, 3, 8, 11, 16, 17). Resistance to azoles has been clearly documented in *Candida* spp. (8, 16, 17, 21) and less frequently in *C. neoformans* (1–4, 22). Although recurrent cryptococcal infection in AIDS patients is usually not associated with the development of azole resistance (3, 4, 13), it may be prudent to monitor recurrent isolates for decreased susceptibility to fluconazole (1–3, 6, 11, 13). To this end, the availability of a reliable method for determining in vitro susceptibility to fluconazole would be useful for both clinical and epidemiological reasons (6, 19).

The microdilution adaptations to the National Committee for Clinical Laboratory Standards (NCCLS) macrodilution reference method have been shown to be useful in testing a broad range of yeast isolates and are now incorporated into NCCLS document M27-A (10). The reference broth microdilution method, which uses RPMI 1640 broth medium, has been used successfully to test isolates of *C. neoformans* (1, 3). There has been concern regarding the use of the NCCLS method for testing *C. neoformans*, because some isolates may grow slowly in the RPMI medium and the recommended incubation time of 72 h has been deemed too long for practical use in the clinical laboratory (5, 6, 19).

Alternative methods for performing in vitro susceptibility testing of *C. neoformans* to fluconazole have been proposed by Kirkpatrick et al. (6) and by Ghannoum and colleagues (5, 19, 22). Kirkpatrick et al. (6) described an agar screening method that broadly categorized isolates as inhibited by $<16$ or $\geq 16$ μg/ml. This approach agrees well with the NCCLS method and provides a means of detecting isolates with decreased susceptibility (MIC $\geq 16$ μg/ml) to fluconazole. Ghannoum described a broth microdilution method that deviated from the NCCLS method by using a slightly higher inoculum ($10^4$ CFU/ml), substitution of yeast nitrogen base (YNB) medium for RPMI 1640 medium, and the use of a spectrophotometer to determine a 50% inhibition endpoint (5, 19). Studies comparing the YNB-based method of Ghannoum and the NCCLS microdilution method for testing *C. neoformans* against fluconazole have shown excellent interlaboratory agreement and approximately 90% agreement with the NCCLS method when testing a small (53 isolates) number of clinical isolates from California (19). Furthermore, the YNB method of Ghannoum has been shown by Witt et al. (22) to be predictive of clinical response in AIDS patients with cryptococcal meningitis who were treated with fluconazole. Given these findings, there is a need for further evaluation of both the YNB method and the NCCLS microdilution method for testing clinical isolates of *C. neoformans* against fluconazole.

In the present study, we have extended the evaluation of the YNB method to include further comparison of this method with the NCCLS microdilution method by using a larger number (149 isolates) of clinical isolates from Ugandan AIDS patients with meningitis in an effort to obtain information on the susceptibility of *C. neoformans* strains from Africa to fluconazole. In the course of this study, we have reevaluated the feasibility of a shorter (48-h) incubation time for the NCCLS method and have examined the clinical correlation of fluconazole MICs determined by both methods with data available for a limited number of isolates and patients.

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TABLE 1. In vitro activity of fluconazole against 149 clinical isolates of *C. neoformans* from Ugandan AIDS patients by two different broth microdilution methods

| Method[a] | MIC (μg/ml)[b] | | | % Agreement[c] |
|---|---|---|---|---|
| | Range | 50% | 90% | |
| NCCLS M27-A | 1.0–16 | 8.0 | 8.0 | |
| YNB | 0.125–16 | 4.0 | 8.0 | 88 |

[a] NCCLS M27-A, reference microdilution method read visually after 48 h of incubation; YNB, microdilution method using yeast nitrogen base medium and spectrophotometric reading at 48 h.
[b] 50% and 90%, MICs for 50% and 90% of isolates tested, respectively.
[c] Percent agreement within 2 $\log_2$ dilutions of the reference MIC.

## MATERIALS AND METHODS

**Isolates and patients.** One hundred and forty-nine clinical *C. neoformans* isolates that were obtained from the cerebrospinal fluid (CSF) samples from 120 Ugandan AIDS patients were used in the study. All patients were treated with fluconazole at doses of 400 to 1,200 mg/day. None of the patients had received fluconazole prior to the diagnosis of cryptococcal meningitis. The isolates were identified as *C. neoformans* var. *neoformans* by standard methods (20), including lack of blue color development on Canavanine-glycine-bromthymol blue agar (7). The isolates were stored frozen at −20°C in 20% glycerol until used in the study.

Clinical outcome data were available for 35 patients with isolates tested by both methods. Patients were considered cured if they were free of signs and symptoms of meningitis and their CSF culture was negative at the end of 10 weeks of fluconazole therapy. Patients with persisting clinical symptoms and positive CSF cultures after 10 weeks of therapy were considered failures. Eleven of the 35 patients (31%) died within the first 10 weeks of therapy (range, 2 days to 5 weeks). CSF cultures remained positive in five (45%) of these patients.

**Antifungal agents.** Fluconazole was provided as a powder by Pfizer Pharmaceuticals Group (New York, N.Y.). A concentrated stock solution (1 mg/ml) was prepared in distilled water and stored at −20°C until used in the study.

**Media.** Two broth media were used: yeast nitrogen base (YNB) medium (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) glucose and buffered to pH 7.0 with 0.05 M morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Company, St. Louis, Mo.) and RPMI 1640 medium (Sigma) with L-glutamine and buffered to pH 7.0 with MOPS buffer (0.165 M).

**Susceptibility testing.** Fluconazole susceptibilities of the 149 *C. neoformans* isolates were determined at the Center for Medical Mycology, Mycology Reference Laboratory, Cleveland, Ohio, by the YNB method of Sanati et al. (19) with YNB medium, a microdilution format, and an inoculum of $10^4$ CFU/ml. The microdilution trays were incubated at 35°C for 48 h. The $A_{420}$ of each well was measured spectrophotometrically, and the MIC at which 50% growth inhibition relative to growth in the drug-free control occurred was determined.

A duplicate set of isolates was tested at the University of Iowa by a broth microdilution method performed as described in NCCLS document M27-A (10) with RPMI 1640 medium and an inoculum of $0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/ml. The microdilution trays were incubated at 35°C and were read after both 48 and 72 h of incubation. The MIC was determined visually according to NCCLS recommendations of 80% reduction in turbidity.

MIC endpoint discrepancies of no more than two dilutions (two wells) were used to calculate percent agreement.

## RESULTS AND DISCUSSION

All 149 isolates of *C. neoformans* grew well in the RPMI 1640 medium, allowing MICs to be determined after both 48 and 72 h by the NCCLS method. The fluconazole MICs determined after 48 h of incubation by the NCCLS method were identical to those determined after 72 h of incubation. Similarly, all isolates grew well in YNB, and MICs were determined after 48 h.

Table 1 summarizes the in vitro susceptibilities of 149 Ugandan *C. neoformans* isolates to fluconazole as determined by the NCCLS and the YNB microdilution methods. As noted previously by Sanati et al. (19), the range of fluconazole MICs obtained with the YNB microdilution method was broader than that obtained with the NCCLS method; however, the modal MICs were within 1 $\log_2$ dilution of one another: 4.0 versus 8.0 μg/ml, respectively. The overall agreement between the two microdilution methods was 88%, a value consistent with that reported previously by Sanati et al. (19).

The overall susceptibility of the Ugandan *C. neoformans* isolates to fluconazole was remarkably similar to that reported for American isolates. The recent study of Kirkpatrick et al. (6) found 92% (84 of 91 isolates) of isolates from Texas and Connecticut to be inhibited by <16 μg of fluconazole per ml. In the present study, 95% of the 149 Ugandan isolates were inhibited by <16 μg/ml irrespective of the testing method.

Clinical data were available for only 35 patients (Table 2). Overall, 45.7% of the patients had clinical improvement and were culture negative at the end of therapy (cured), 22.8% failed therapy, and 31% died prior to completing the full course of therapy. These data are not dissimilar to those reported from clinical trials in the United States, where clinical success ranged from 40 to 60% with various treatment regimens (9).

Given the small number of patients, our ability to make any conclusions regarding the clinical value of the susceptibility data generated by the two methods is extremely limited. Nevertheless, it appears that the broader range of MICs generated by the YNB method may have some value (Table 2). Of the 16 patients infected with a strain of *C. neoformans* inhibited by ≤2.0 μg of fluconazole per ml as determined by the YNB method, 9 (56%) were considered therapeutic successes (cured), whereas only 7 of 19 patients (37%) infected with strains inhibited by ≥4.0 μg/ml had a favorable outcome. Notably, 7 of the 11 early deaths (64%) occurred among patients infected with strains inhibited by ≥4.0 μg/ml. In contrast, the NCCLS method provided little discrimination among the 35 isolates, with 32 (91%) inhibited by either 4.0 or 8.0 μg of fluconazole per ml. Forty-four percent of patients infected with isolates inhibited by ≤4.0 μg/ml were cured versus 47% of patients infected with isolates inhibited by ≥8.0 μg of fluconazole per ml as determined by the NCCLS method.

The results of this study confirm and extend the previous observations of Sanati et al. (19) and of Witt et al. (22). We found that although the YNB method agrees reasonably well with the reference microdilution method, it provides a broader range of MICs, with several isolates inhibited by lower concentrations of fluconazole than was apparent with the NCCLS method. The NCCLS method tended to bunch isolates together with MICs of 4.0 and 8.0 μg/ml. This greater degree of

TABLE 2. Clinical correlation of fluconazole MICs determined by two different methods for testing *C. neoformans* isolates from 35 Ugandan AIDS patients with meningitis[a]

| Fluconazole MIC (μg/ml) | No. of patients with the indicated clinical outcome by test method | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | YNB[b] | | | | NCCLS M27-A[c] | | | |
| | Tested | Cured | Failed | Died[d] | Tested | Cured | Failed | Died |
| 0.5 | 2 | | | 2 | | | | |
| 1.0 | 5 | 3 | 1 | 1 | | | | |
| 2.0 | 9 | 6 | 2 | 1 | 1 | | | 1 |
| 4.0 | 11 | 3 | 4 | 4 | 15 | 7 | 5 | 3 |
| 8.0 | 8 | 4 | 1 | 3 | 18 | 8 | 3 | 7 |
| 16 | | | | | 1 | 1 | | |

[a] All patients were treated with fluconazole at doses of 400 to 1,200 mg/day.
[b] YNB, microdilution method using yeast nitrogen base medium and spectrophotometric reading at 48 h.
[c] NCCLS M27-A, reference microdilution method read visually after 48 h of incubation.
[d] Patients who died within the first 10 weeks of therapy (five with positive CSF cultures).

discrimination among isolates may be important, as it appears, given very limited data, that the clinical outcome may be better among patients infected with strains inhibited by lower concentrations of fluconazole as determined by the YNB method. A similar trend was observed by Witt et al. (22).

It should be emphasized that this collection of isolates was quite susceptible to fluconazole, irrespective of the method used for testing (Table 1). None of the isolates required more than 16 μg of fluconazole per ml for inhibition and only 8% were inhibited by 16 μg/ml by either test method. Given the overall susceptible nature of the isolates, it may not be possible for one testing method to predict treatment outcome better than any other. Doses of fluconazole of ≥400 mg/day will routinely provide serum and CSF concentrations well in excess of 32 μg/ml (18). Thus, the clinical response observed is more likely to be influenced by the profound nature of the patient's immunosuppression than by the fluconazole MIC as determined by any given test method. Of course, the situation may be quite different when patients are infected with strains requiring more than 16 μg of fluconazole per ml for growth inhibition. Such isolates are quite unusual in our experience (14). Detailed analysis of patients infected with strains of *C. neoformans* with decreased susceptibility to fluconazole should prove instructive and provide information that will be useful in further assessment of the two test methods and for the eventual establishment of interpretive breakpoints.

In summary, we have shown that isolates of *C. neoformans* from Ugandan AIDS patients are quite susceptible to fluconazole as determined by both YNB and NCCLS microdilution methods. Fluconazole MICs can be determined by the NCCLS method as well as the YNB method after 48 h of incubation, and the values obtained by both methods are in close agreement. The extended range of MICs provided by the YNB method may be of clinical value in that it appears that those individuals infected with *C. neoformans* strains that are inhibited by lower concentrations of fluconazole respond somewhat better to treatment than those infected with strains requiring higher concentrations. Thus, the YNB method is a viable option for testing *C. neoformans*. The value of this or any other method of in vitro susceptibility testing of *C. neoformans* in directing therapy in the treatment of AIDS patients with cryptococcal meningitis remains to be determined and will require further study with larger numbers of patients and isolates.

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